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EFFECT OF OXYGEN ON THE CAPACITY OF CARBON DIOXIDE-FIXATION BY GREEN ALGAE

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(Received for publication, January 31, 1955)

The fact that molecular oxygen, when applied in a relatively large amount, has an inhibitory effect upon photosynthesis was first discovered by Warburg (*1*, *2*) and later confirmed by Wassink *et al.* (*3*) as well as by Tamiya and Huzisige (*4*). The last named authors have found (i) that the inhibition is "reversible", *i.e.*, it is completely eliminated when oxygen is removed, and (ii) that the inhibition is of "second order", which means that the following relation holds between the degree of inhibition (defined by $H=1-v/v_N$, v and v_N being the rate of photosynthesis in the presence and absence, respectively, of oxygen) and the oxygen tension applied:

$$H=\frac{[O_2]^2}{\Phi^2+[O_2]^2} \tag{1}$$

where Φ is a constant corresponding to the oxygen tension causing 50 per cent inhibition. Based on the observation that the inhibition became stronger when the concentration of CO_2 supplied was lowered, it was inferred that the site of attack by oxygen may be some step involved in the process of CO_2-uptake.

Apart from these observations it was found by Mehler *et al.* (*5*, *6*, *7*) that oxygen can react as an oxidant in the Hill reaction. This finding seems to throw light on the mechanism of inhibitory action of oxygen upon photosynthesis, since, as is generally conceded now, the action of the Hill reagents lies in the oxidation of photochemically produced reducing substance which, in normal photosynthesis, reduces the substance derived from CO_2.

As the reducing substance to be produced in the primary phase of photosynthesis we may envisage the CO_2-fixing agent, whose existence in illuminated green cells was first demonstrated by Calvin and Benson (*8*) using radioactive carbon as a tracer. It appears, therefore, worth while to investigate whether the reducing capacity in the sense of Calvin and Benson is affected by the presence of oxygen. Regarding the possible mechanism of inhibitory action of oxygen upon photosynthesis, Warburg had made the following remarks in his pioneer work (*2*) published in 1920: "Was die Deutung der Versuche betrifft, so kommen zunächst zwei Möglichkeiten in Betracht. Bei hohen Bestrahlungsintensitäten entstehen die Assimilate in hoher Konzentration und könnten, ehe sie in eine stabile Form übergehen, durch Sauerstoff zu Kohlensäure zurückoxydiert werden. Dies wäre der uninteressantere Fall. Es ist aber auch daran zu denken, dass der Sauerstoff mit dem photochemischen Primärprodukt reagiert, indem er, wie das Kohlensäurederivat, als Acceptor fungiert".

The work that is reported below will show that oxygen inhibits photosynthesis by consuming the primary reducing substance in the sense of Calvin and Benson, and indeed exactly in the manner which was thought by Warburg to be the more interesting of the two possible mechanism he mentioned.

MATERIAL AND METHODS

Experimental material was the dark cells* of *Chlorella ellipsoidea* which were grown according to the method of Tamiya *et al.* (*9*). The cells, after being washed twice with $M/500$ K_2SO_4 solution, were suspended, in a concentration of about 6 ml. (packed volume) per litre, in $M/50$ phosphate buffer of pH 7. The $C^{14}O_2$-fixing power of cells was determined according to the principle of Benson *et al.* (*10*) using two kinds of apparatus which are illustrated in Figs. 1 and 2. Both have a round flat vessel, "lollipop" (*A*), at the top, and one or four "dark vessels" (*D*) at the bottom. For convenience of reference we may call these two devices Apparatus I and II, respectively. The lollipop is the vessel in which the cells were pre-treated (in the absence of $C^{14}O_2$) under certain conditions, and the dark vessels are the chambers in which the pre-treated cells were brought into contact with $C^{14}O_2$ in the dark. Between the lollipop and the dark vessel(s), there is a space *C* in Apparatus I, and *B* and *C* in Apparatus II, the capacity of *C* being 3.2 ml. in Apparatus I, and 1.8 ml. each in Apparatus II. The lollipop (55 ml. in volume and 2 cm. in thickness) was provided with a tube for flushing gases (N_2, O_2 or their mixture) in the cell suspension, and according to the experimental purposes, it might be either kept dark or illuminated from both sides with tungsten lamps of 200 watts each. The effective light intensity applied in the illuminating experiments was about $2 \times 10{,}000$ lux.

Apparatus I had several exchangeable dark vessels and was used to study the time course of the effect of pre-treatment of cells, the duration and conditions of

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* For the meaning of this terminology see Tamiya *et al.* (*9*).

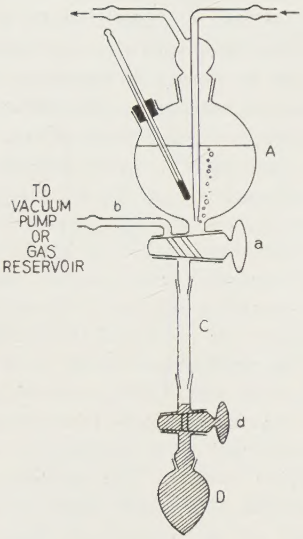

FIG. 1. Apparatus (I) used for studying the time course of the effect of pre-treatment of cells. Explanation in text.

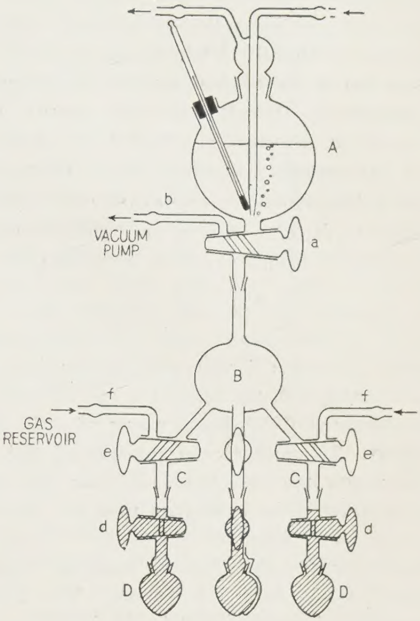

FIG. 2. Apparatus (II) used for studying the time course of the reaction of cells with $C^{14}O_2$ in the dark. Explanation in text.

subsequent reaction of cells with $C^{14}O_2$ in the dark vessels being held the same. Apparatus II was used to investigate the time course of the reaction of cells with $C^{14}O_2$ in the dark vessels, the condition and duration of pre-treatment being kept the same.

To begin with, 40–60 ml. of cell suspension was placed in the lollipop, stopcocks *a*, *d* and *e* (in Apparatus II) being set in such a way as to connect the dark vessel(s) —through *C* or *C* and *B*—to tube *b*. On the other hand, the dark vessel(s) was provided with 0.1 ml. each of $NaHC^{14}O_3$-solution (0.03 *M*). When the pre-treatment of algal suspension in the lollipop (aeration with appropriate gas[*] with or without illumination) proceeded for a certain period of time, all parts except the lollipop were evacuated through tube *b*. Then, stopcock(s) *d* was closed and, by turning the direction of stopcock *a*, all (in Apparatus II) or a part (in Apparatus I) of the algal suspension in the lollipop was introduced into space *B* and *C* (Apparatus II) or *C* (Apparatus I). Immediately after space(s) *C* was fully filled with the algal suspension, it was connected—by tube *b* in Apparatus I and by tubes *f* in Apparatus II—to a gas reservoir (not shown in the figure), in which the same gas as flushed in the lollipop was contained, and at the same time stopcock(s) *d* was opened so that the content of *C* was completely transferred into each dark vessel* by the pressure of gas from the reservoir. After allowing the cells to react with $C^{14}O_2$ for a certain period of time, each dark vessel was removed—one by one in Apparatus II—from the apparatus and the cells contained in it were immediately killed by introducing 0.5 ml. of concentrated acetic acid-hydrochloric acid-mixture (4 : 1) into the vessel. When Apparatus I was used, a new dark vessel containing the $NaHC^{14}O_3$-solution was connected to the apparatus and the same procedure was conducted to determine the change of $C^{14}O_2$-fixing power of cells during the pre-treatment going on in the lollipop. To measure the quantity of C^{14} fixed by the cells, 1.8 ml. each of algal cells, which had been killed with the acid mixture, was transferred to a dish of stainless steel, dried by irradiation with an infrared lamp, and their radioactivity was determined by using an end-window Geiger-Müller counter. The procedure of transferring the algal samples from the lollipop into the dark vessels could be conducted within only 2–4 seconds. The temperature of the experiment, from the beginning of pre-treatment in the lollipop until the killing of cells with acid mixture, was 20° or 25° in the present study.

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[*] In each experiment, prior to the main pre-treatment, the algal suspension was first evacuated, and then kept in the dark for 20 minutes under continuous bubbling of the gas which was to be flushed in the pre-treatment in the main experiment. When, during the course of pre-treatment, the flushing gas was replaced with some other gas, the algal suspension was promptly evacuated before the second gas was introduced.

PRELIMINARY EXPERIMENTS

It has been shown by Calvin and Benson (*8*) that the activity of algal cells (*Chlorella* and *Scenedesmus*) to fix $C^{14}O_2$ in the dark was greatly enhanced when the algae were illuminated prior to the provision of $C^{14}O_2$. They also found that the light-induced capacity of dark $C^{14}O_2$-fixation rapidly decreased when the light was turned off. In these experiments the algal cells were kept in a helium atmosphere before they were brought into contact with $C^{14}O_2$.

Before studying the effect of O_2 on these processes, we repeated the experiment of the American authors (using nitrogen instead of helium) and have wholly corroborated their statements. The results of our experiments are reproduced in Figs. 3 and 4. In the experiment

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* The final concentration of $NaHC^{14}O_3$ in the mixture thus formed in each dark vessel was $1.0\sim1.7\times10^{-3}\,M$, which was confirmed to be saturating for the dark $C^{14}O_2$-fixation.

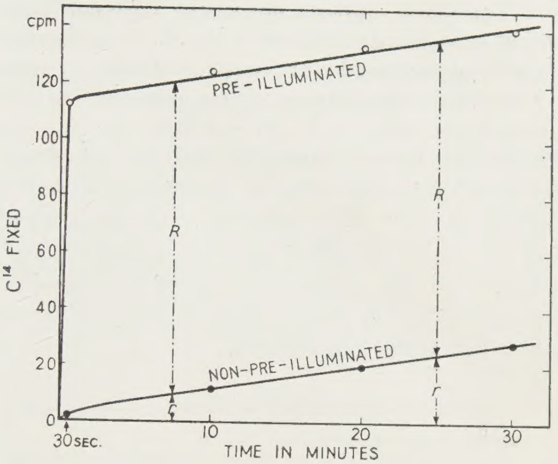

FIG. 3. Time courses of dark $C^{14}O_2$-fixation in N_2 by sufficiently pre-illuminated and non-pre-illuminated cells (Apparatus II; temp. 25°).

Pre-illumination lasted for 30 minutes, which was of sufficient length to bring about the full effect of pre-illumination. *R* indicates the "light-induced reducing power", and *r* the "background (or dark) reducing power". Ordinate: count per minute (cpm) of C^{14} fixed in the dark; abscissa: the time of contact of cells with $C^{14}O_2$ in the dark.

shown in Fig. 3, the algal samples, one kept illuminated and the other kept in the dark, were flushed with N_2 for more than 30 minutes.* The algae were then promptly introduced to the dark vessels, and at different times (30 seconds, 20, 40 and 60 minutes) after contact with $C^{14}O_2$, aliquots were tested for their radioactivity. As may be seen from the figure, the radioactivity of non-illuminated cells increased slowly, and indeed almost linearly with the time of contact of cells with $C^{14}O_2$, whereas that of pre-illuminated cells increased steeply at first, and after attaining a certain level, its increase abruptly slowed down, showing thereafter a steady rate that was practically the same as that found for non-illuminated cells. At each time on the abscissa of the figure, the vertical distance between the two curves represents the reducing power produced by the effect of light. In the following, this increment of $C^{14}O_2$-fixing capacity caused by light shall be called "light-induced reducing power" and denoted by R. On the other hand, the capacity of the "background (or dark) reducing power"—which is denoted by r in the following—may be assessed by the radioactivity fixed within a definite time (*e.g.* 20 minutes) by non-pre-illuminated cells. As exemplified by the curves in Fig. 3, the $C^{14}O_2$ fixed within 30 seconds by pre-illuminated cells is practically equal to the "light-induced reducing power" (R), since the "background reduction" (r) effected during the same period of time by non-pre-illuminated cells is almost negligible. For this reason, in most of the experiments described below, the amount of $C^{14}O_2$ fixed within 30 seconds by pre-illuminated cells was regarded as being equal to R.

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* As will be shown later, illumination for 30 minutes was practically of sufficient length to bring about the full effect of pre-illumination.

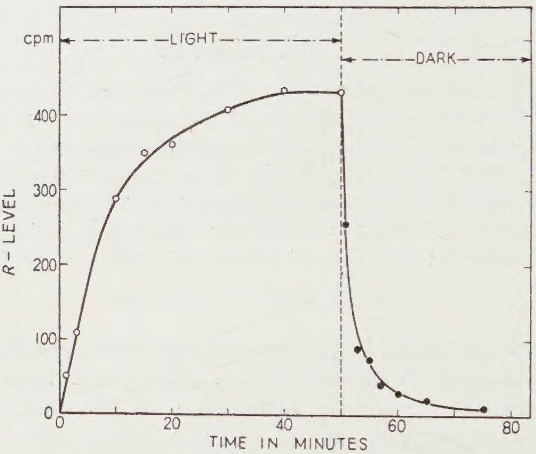

FIG. 4. Formation of "light-induced reducing power" (R) in the light and its decay in the dark under anaerobic condition (Apparatus I; temp. 20°).

Ordinate: cpm of C^{14} fixed in 30 seconds in the dark; abscissa: the time which the cells spent in the light and dark, as indicated, prior to the provision of $C^{14}O_2$.

The process of formation of R in the light and its decay in the dark was studied using N_2 as flushing gas. The results obtained are reproduced in Fig. 4. It may be seen that, when the cells were illuminated, R (measured by 30-seconds' $C^{14}O_2$-fixation) increased at first rapidly and later more slowly to attain a stationary value in about 40 minutes.

When at this stage the light was turned off, R decreased very rapidly to become almost zero in about 30 minutes.

Effect of Oxygen on the "Background Reducing Power" Shown by Non-pre-illuminated Cells

Before presenting the data dealing with the effect of oxygen upon the light-induced reducing power (R), it seems relevant to describe our observations on the effect of oxygen upon the background reducing power (r) which was measured by the radioactivity fixed in 20-minutes' contact with $C^{14}O_2$ by non-illuminated cells. The r-value thus measured was found to be more or less increased by the effect of oxygen and, what was remarkable, the degree of increase was largely dependent on the duration of contact of cells with oxygen. The situation is clearly demonstrated by the experiment reproduced in Fig. 5. In this experiment, the algal suspension was first aerated with N_2, and at the expiration of 20 minutes the nitrogen was promptly replaced with oxygen, all procedures being carried out in the dark. At various intervals during this process, aliquots of algal suspension were brought into contact with $C^{14}O_2$ in the dark, and the radioactivity fixed in 20 minutes was meas-

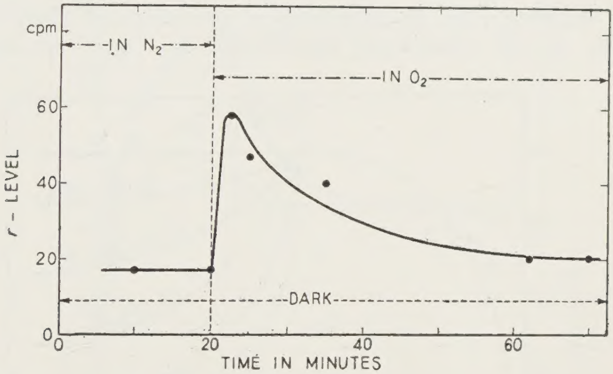

FIG. 5. Dark $C^{14}O_2$-fixation (in 20 minutes) by algal cells that have been kept dark, first in N_2 and later in O_2 atmosphere (Apparatus I; temp. 25°).

Ordinate: cpm of C^{14} fixed in 20 minutes' contact with $C^{14}O_2$ in the dark; abscissa: time of pre-incubation in the dark with flushing of N_2 and O_2 as indicated.

ured. It is apparent from the figure that when the cell suspension was transferred from N_2- to O_2-atmosphere, there occurred an abrupt increase in the *r*-value, which, however, soon tapered off and attained eventually a certain steady level. The final *r*-value established in O_2 atmosphere was always higher than the steady *r*-value obtained in N_2-atmosphere. It may be seen from this experiment that the formation of dark reducing power (*r*) is a process that is closely related to the respiratory metabolism of algal cells. The remarkable temporary increase of *r*-value at the transition from anaerobiosis to aerobiosis may probably be due to the situation that the prolonged anaerobiosis causes the accumulation in algal cells of some substance which, when contact with O_2, is immediately changed into an acceptor of CO_2.

Effect of Oxygen on the Level of Light-induced Reducing Power

As shown in a previous experiment (Fig. 4), the value of *R* attains a certain stationary level on prolonged illumination of algal cells in N_2 atmosphere. In the experiment reproduced in Fig. 6 we investigated

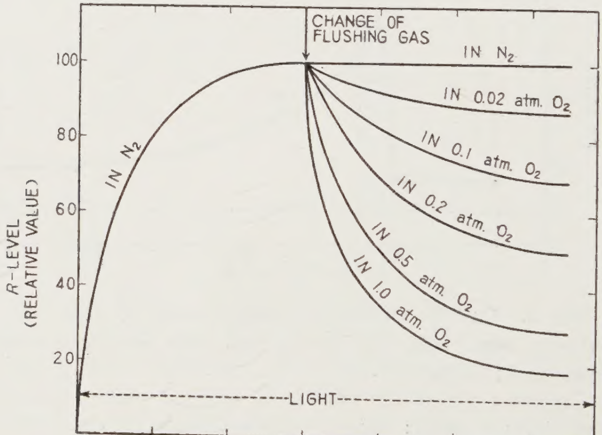

FIG. 6. Effect (in the light) of varying concentrations of O_2 on *R* preformed in N_2 atmosphere (Apparatus II; temp. 25°).

Ordinate: relative amount of C^{14} fixed in 30 seconds in the dark. Abscissa: the time which the cells spent in the light (prior to the provision of $C^{14}O_2$), first in N_2 atmosphere, and later in various gas mixtures as indicated.

whether this stationary level (established in the light) was modified by the effect of oxygen. The cells were illuminated, first in N_2 atmosphere, for 30 minutes—by which time *R* attained the stationary (maximum) level—and then, under continued illumination, nitrogen was replaced by gas mixtures containing varying concentrations of oxygen. At intervals during this procedure, the *R*-level was determined by measuring the radioactivity fixed in 30 seconds of contact of cells with $C^{14}O_2$ in the dark.

As is apparent from the figure, the *R*-level is markedly susceptible to oxygen, and indeed the higher the O_2 concentration in the gas mixture applied, the more pronounced was the decrease of the stationary *R*-level. In all cases the new stationary levels of *R* were established in about 30 minutes' contact of cells with O_2-containing atmosphere.

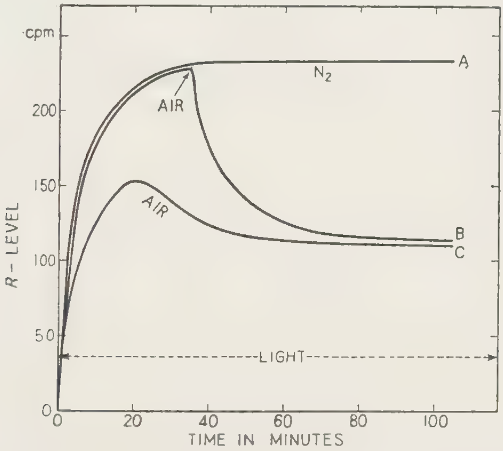

FIG. 7. Effect of air on the time course of *R*-formation in the light (Apparatus I; temp. 20°).

Ordinate: cpm of C^{14} fixed in 30 seconds in the dark. Abscissa: the time which the cells spent in the light (prior to the provision of $C^{14}O_2$) under various conditions as indicated.

Curve A: algal suspension was flushed with N_2 throughout the experiment.

Curve B: algal suspension was flushed first with N_2 and later (at the time indicated by the arrow) with air.

Curve C: algal suspension was flushed with air from the beginning of illumination.

It may be remarked that the final stationary *R*-level in the presence of oxygen is quite independent of the time of provision of O_2-containing gas. In the experiment illustrated in Fig. 7, a cell suspension was flushed with air (20 per cent O_2) from the beginning of illumination; another algal suspension was first flushed with N_2 which was later replaced by air. As may be seen from the figure, the final stationary *R*-level attained were the same in both cases. It should be noticed that at the initial phase of ascension all curves coincided with each other, indicating that the process of formation of *R* was not affected by oxygen.

All the *R*-values measured in the above experiments were determined by the radioactivity fixed in 30 seconds' contact of pre-illuminated cells with $C^{14}O_2$ in the dark. In the experiment given in Fig. 8, the cells were pre-illuminated in N_2 or O_2 for 30 minutes and then, after transferring the cells into dark vessels, the time course of $C^{14}O_2$-fixation in the dark was traced. As is apparent from the figure, it is only the initial (30-seconds') $C^{11}O_2$-pickup that was suppressed by the effect of oxygen. A peculiar phenomenon was observed, however, when we followed the effect of O_2 (given during the period of pre-illumination),

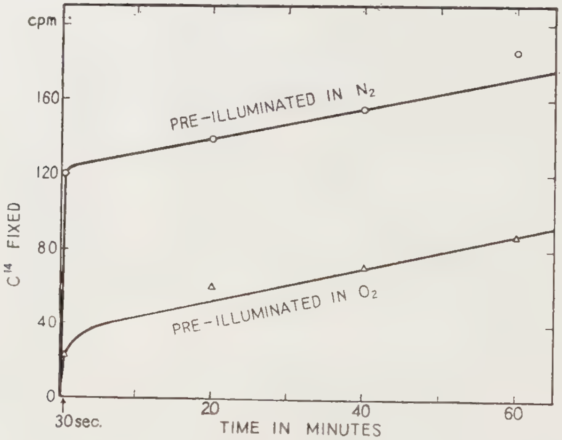

FIG. 8. Time course of dark $C^{14}O_2$-fixation by algal cells which were pre-illuminated for 30 minutes in N_2- and O_2-atmosphere, respectively (Apparatus II; temp. 25°).

Ordinate: cpm of C^{14} fixed in the dark. Abscissa: the time of contact of cells with $C^{14}O_2$ in the dark.

not by determining the 30-seconds' $C^{14}O_2$-fixation, but by measuring the radioactivity fixed in 20 minutes' contact (in the dark) of cells with $C^{14}O_2$. An experiment similar to those shown in Fig. 6 was performed using the same algal sample as applied in the experiment presented in Fig. 5. The algal suspension which had been kept illuminated in N_2 atmosphere for 30 minutes was abruptly exposed to pure O_2, the illumination being continued as ever. At various intervals from the beginning of illumination in N_2, aliquots of algal samples were introduced into the dark vessel, and the capacity of $C^{14}O_2$-fixation was determined by measuring the radioactivity *fixed in 20 minutes.* The results obtained are reproduced by Curve A in Fig. 9. It may be seen that immediately after the replacement of N_2 by O_2, the curve showed a conspicuous upswing, after which it abruptly descended to attain eventually a steady value. As pointed out already, the radioactivity fixed in prolonged contact of pre-illuminated cells with $C^{14}O_2$ in the dark represents

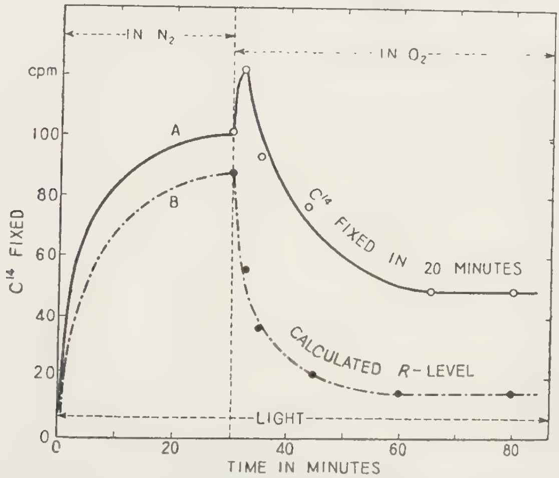

FIG. 9. Twenty minutes' $C^{14}O_2$-fixation by algal cells that have been pre-illuminated first in N_2 -and later in O_2-atmosphere (Apparatus I; temp. 25°).

Curve A: $C^{14}O_2$ fixed in 20 minutes in the dark.

Curve B: values of *R* obtained by subtracting the data of experiment shown in Fig. 5 from the values represented by Curve A. Abscissa: the time which the cells spent in the light (prior to the provision of $C^{14}O_2$) first in N_2 and later in O_2 as indicated.

the sum of *R* and the "background $C^{14}O_2$-fixation" (*r*) occurring in the meantime. Since the *r*-value obtained in "20-minutes' dark $C^{14}O_2$-fixation" by non-pre-illuminated cells had been measured in the experiment given in Fig. 5, we may gauge the *R*-value by subtracting the data in Fig. 5 from the data represented by Curve A in Fig. 9.* The values obtained by this subtraction are shown by Curve B in Fig. 9, which, as has been expected, was virtually the same as the curve of *R*-level (for 100 per cent O_2) measured by 30-seconds' $C^{14}O_2$-fixation in the dark (*cf.* Fig. 6).

Reversibility of the Effect of Oxygen on the Level of R in the Light

The suppressing effect of O_2 on the *R*-level established in the light was found to be of reversible nature. This is clearly demonstrated by the data presented in Fig. 10. In this experiment, algae were illuminated, first in N_2 and then in O_2, and when the *R*-value attained the

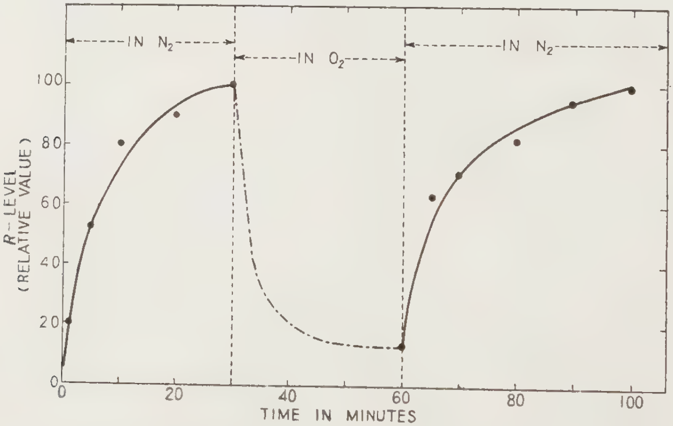

FIG. 10. Reversibility of the effect of oxygen on the level of *R* in the light (Apparatus I; temp. 25°).

Ordinate: cpm of C^{14} fixed in 30 seconds in the dark. Abscissa: the time which the cells spent in the light (prior to the provision of $C^{14}O_2$) in N_2- and O_2-atmosphere as indicated.

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* This subtraction is justified, since these two experiments were performed with entirely the same arrangement: same algal sample, same $NaHC^{14}O_3$-solution, same temperature, same length of $C^{14}O_2$ supply etc.

stationary low level, O_2 was replaced by N_2. As may be seen from the figure, the R-value increased again to attain eventually the original stationary level that has been observed in N_2-atmosphere. Noteworthy is the fact that the recovery of R-level on subsequent provision of N_2 proceeded in exactly the same manner as the initial phase of its formation in N_2-atmosphere. In both cases the stationary level was attained in about 30 minutes of illumination.

Functional Relationship Between the Partial Pressure of Oxygen and the Final R-level Established in the Light

In view of the reversibility of the effect of O_2 upon the R-level, it seems worth while to make clear the functional relationship between the partial pressure of O_2 and the final R-level established in the light. Let us define the degree of suppression of R caused by oxygen by

$$h = 1 - \frac{[R]_S}{[R]_S^N}$$

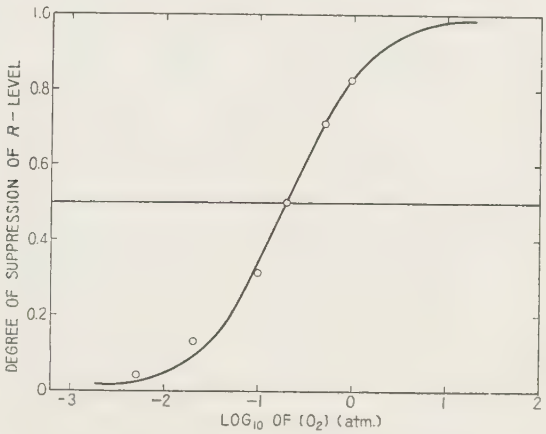


FIG. 11. Functional relationship between the partial pressure of O_2 and the degree of suppression of the final R-level established in the light.

Open circles: degree of suppression (h) of R-level at various partial pressures of O_2 according to the data in Fig. 6; $h=1-[R]_S/[R]_S^N$, where $[R]_S$ and $[R]_S^N$ are the final R-level in the presence and absence, respectively, of O_2. Continuous curve: the values of h calculated from Eq. (2) taking the value of ϕ as 0.22 atm.

where $[R]_S$ and $[R]_S^N$ represent the *final* R-level in the presence and absence, respectively, of oxygen. Inspection of the data presented in Fig. 6 showed that the following relation holds between h and the partial pressure of oxygen applied:

$$h=\frac{[O_2]}{\phi+[O_2]} \qquad (2)$$

where ϕ is a constant corresponding to the oxygen partial pressure causing 50 per cent suppression. In Fig. 11 the open circles represent the observed values of h, and the curve refers to the values of h which were calculated by Eq. (2) taking the value of ϕ as 0.22 atm. The correspondence between the observed and calculated values is satisfactory.

Effect of $C^{14}O_2$ Concentration upon the Suppression of the Final R-level Caused by Oxygen

The effect of O_2 upon the final R-level in the light was found to be modified, to some extent, by the concentration of $C^{14}O_2$ applied in the dark. The experiment was carried out by flushing the algal suspension with N_2 or O_2 for 30 minutes in the light, and by subsequently providing (in the dark) the $NaHC^{14}O_3$-solutions of different concentrations. The stationary R-level was measured by the radioactivity fixed in 30 seconds' contact of cells with $C^{14}O_2$. From the data summarized in Table I, it may be seen that the suppressing effect of O_2 upon stationary R-level is stronger in lower concentrations of $C^{14}O_2$.

TABLE I

Effect of $C^{14}O_2$ Concentration upon the Suppression of the Final R-level Caused by O_2

Concentration of $NaHC^{14}O_3$	Stationary R-level (cpm)		Degree of suppression caused by O_2
	in N_2	in O_2	
5.4×10^{-4} M	1883	340	0.82
5.4×10^{-5} ,,	1029	170	0.83
1.8×10^{-5} ,,	443	49	0.89
6.0×10^{-6} ,,	173	17	0.90

Effect of Oxygen on the Decay in the Dark of R-level

In most of the experiments described above, oxygen was supplied to algal cells that were kept illuminated, and its effect on the *R*-level in the light was investigated. It is now of interest to examine whether, and in what manner, the process of decay of *R* in the dark, as it was observed in the experiment of Fig. 4, will be affected by oxygen. Algae were illuminated in N_2-atmosphere for 50 minutes, by the end of which a stationary *R*-level was fully established. Then, the light was turned off simultaneously with prompt replacement of N_2 with O_2, and the time course of decay of *R*-level was followed. As the control the decay of *R* in N_2-atmosphere was also traced. In Fig. 12 are reproduced the results obtained, from which it may clearly be seen that the rate of decay of *R* in the dark was considerably accelerated by the effect of oxygen.

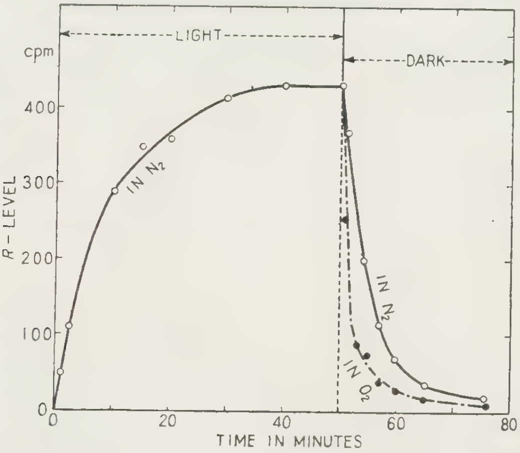


FIG. 12. Effect of O_2 on the decay of *R*-level in the dark (Apparatus I; temp. 20°).

Ordinate: cpm of C^{14} fixed in 30 seconds in the dark. Abscissa: the time which the cells spent in the light and dark, and in N_2 and O_2, as indicated.

Effect of Oxygen on the Normal Photosynthesis Measured by the Fixation of $C^{14}O_2$

Using $C^{14}O_2$ as a tracer we also investigated the effect of O_2 upon normal photosynthesis. Suspension of algae in $M/100$ phosphate buffer (pH 7) was illuminated with 20,000 lux light under constant flushing of N_2, air, 50% N_2+50% O_2 or 100% O_2 (concentration of algae: 0.06 ml. packed volume per liter). At the expiration of 30 minutes, $NaHC^{14}O_3$ (end concentration: 1.5×10^{-3} M) was added to the suspension with continued illumination, and at intervals of 3 minutes aliquots of algal suspension were taken out, killed in acid, and the radioactivity fixed was determined (temp.: 25°). Measurement of $C^{14}O_2$-fixation was continued 15 minutes after the addition of $NaHC^{14}O_3$, during which time the photosynthetic C^{14} fixation proceeded at steady rates which were found to be distinctly lower in the presence of increasing concentration of O_2. The "degree of inhibition" (H) in function of oxygen partial pressure applied is shown in Fig. 13, in which the curve represents the H-values that were calculated by Eq. (1) taking the value of Φ as 1.0 atm. It is apparent that the inhibition was of "second order" in conformity with the results of manometric measurements made by Tamiya *et al.* (*4*).

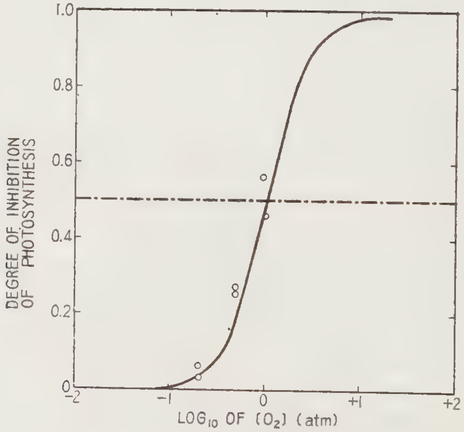


FIG. 13. Functional relationship between the partial pressure of O_2 and the degree of suppression of the normal photosynthesis measured by the amount of $C^{14}O_2$-fixation.

The curve represents the values of H calculated from Eq. (1) taking the value of Φ as 1.0 atm.

Theoretical Considerations

All the experiments described above show indubitably that the R-level produced by light in algal cells is strongly decreased by the effect of oxygen. In the following we shall attempt to propose a picture of mechanism which may underlie various phenomena we have observed. In the first place we shall assume that the R-level observed in our experiments corresponds to the concentration of a certain substance R which is produced photochemically from its precursor (Q) by a reaction such as

$$Q \xrightarrow{k_i} R \qquad (3)$$

where k_i is the rate constant of the reaction. Conceivably, this reaction is accompanied by the evolution of oxygen. Characteristic behavior of substance R is that it spontaneously changes into an inactive form. The inactivation may be supposed to be due to the back-reaction of R to Q, thus:

$$R \xrightarrow{k'} Q \qquad (4)$$

where k' is the rate constant. Another characteristic of R is its reactivity with O_2 to form inactive products. This reaction may be supposed to be

$$R+O_2 \xrightarrow{k''} X \quad \text{or} \quad R+O_2 \xrightarrow{k''} Q+X \qquad (5)$$

where X represents an unknown product or products, and k'' the reaction constant. It is postulated that the Hill reaction caused by O_2 is a result of Reactions (3) and (5). It is further assumed that the substance Q exists in the cell in a sufficiently large amount, so that its concentration does not change with the formation of R.

From Reactions (3), (4) and (5) we have

$$\frac{d[R]}{dt}=k_i[Q]-k'[R]-k''[O_2][R] \qquad (6)$$

Let us now consider the process of formation of R in illuminated algal cells which have been kept dark for a sufficiently long time prior to the illumination. Considering that $[R]=0$ at $t=0$ (t being the time of illumination), we obtain the following equation by integrating Eq. (6):

$$[R]=\frac{k_i[Q]}{k'+k''[O_2]}(1-e^{-(k'+k''[O_2])t}) \qquad (7)$$

In the absence of O_2:

$$[R]=\frac{k_i[Q]}{k'}(1-e^{-k't}) \tag{8}$$

If we denote by $[R]_0$ the R-level at the very beginning of illumination, we have from Eq. (7)

$$[R]_0=k_i[Q]t \tag{9}$$

If, on the other hand, the stationary R-level which obtains on sufficiently long illumination is denoted by $[R]_S$, we have from Eq. (7)

$$[R]_S=\frac{k_i[Q]}{k'+k''[O_2]} \tag{10}$$

In the absence of oxygen:

$$[R]_S^N=\frac{k_i[Q]}{k'} \tag{11}$$

In the experiment reproduced in Figs. 6 and 11 the "degree of suppression" (h) of $[R]_S$ in function of $[O_2]$ was determined. Considering Eqs. (10) and (11) we have

$$h=1-\frac{[R]_S}{[R]_S^N}=\frac{[O_2]}{\frac{k'}{k''}+[O_2]} \tag{12}$$

This formula agrees with Eq. (2) which has been obtained by experiment, and shows that the value ϕ is nothing but the ratio of k' to k'':

$$\phi=\frac{k'}{k''} \tag{13}$$

In the normal mechanism of photosynthesis, R reacts with CO_2, or more probably, with a substance or substances derived from CO_2. If we denote such a substance by C, the reaction between R and C may be supposed to be something like:

$$R+C\xrightarrow{k_1}Q+x \tag{14}$$

where x is a reduction product formed from C. In the presence of CO_2 (during illumination), the rate of change of R is given by

$$\frac{d[R]}{dt}=k_i[Q]-k'[R]-k''[O_2][R]-k_1[C][R]$$

Under such a condition, the relation corresponding to Eq. (12) will assume the form:

$$h=\frac{[O_2]}{\frac{k'+k_1[C]}{k''}+[O_2]} \tag{15}$$

The validity of this equation cannot be tested by our experimental data, since we could not measure the R-level with the provision *in the light* of CO_2. In the experiment given in Table I, the final R-level established in N_2- and O_2-atmospheres were measured by applying *in the dark* different concentrations of $NaHC^{14}O_3$. Obviously, the state of affairs in this experiment was not the same as that considered above. Nevertheless, we may suppose that during the 30-seconds' dark period of $C^{14}O_2$-provision, there had occurred a competition between O_2 and C for R as it is represented by Eqs. (5) and (14). Our finding in Table I that the suppressing effect of O_2 upon the stationary R-level was stronger in lower $C^{14}O_2$-concentration may, therefore, be taken as reflecting the situation represented by Eq. (15), according to which the decrease of CO_2-concentration will render ϕ to become smaller, or the inhibition caused by a given concentration of O_2 to become stronger.

Considering Reactions (4) and (5) the rate of decay of the R-level in the dark is given by

$$\frac{-d[R]}{dt}=k'[R]+k''[O_2][R] \tag{16}$$

where t is the time elapsed after cessation of illumination. In the experiment given in Fig. 12 the concentration of R at $t=0$ was $[R]^N_S$ (see Eq. 11). With this in view, we obtain by integrating Eq. (16):

$$[R]=[R]^N_S e^{-(k'+k''[O_2]t)} \tag{17}$$

or in the absence of oxygen:

$$[R]=[R]^N_S e^{-k't} \tag{18}$$

By applying Eqs. (9) and (11) to the experimental data presented in Fig. 12, the values of $[R]^N_S$, $k_i[Q]$ and k' were estimated to be as follows:

$$\left.\begin{array}{l}[R]^N_S=425 \text{ cpm.}\\ k_i[Q]=60 \text{ cpm./min.}\\ k'=0.14 \text{ 1./min.}\end{array}\right\} \tag{19}$$

From the data given in Fig. 11, ϕ was found to be 0.22 atm. Substituting these values of ϕ and k' into Eq. (13), k'' is evaluated to be

$$k''=0.64 \text{ l./min.-atm.} \tag{20}$$

It should be remarked that the values of $k_i[Q]$ and $[R]_S^N$ mentioned above contain the term of cpm in their dimension, and, therefore, are subject to change according to the arrangement of experiment (concentration of algal cells used and the concentration of $C^{14}O_2$ in the solution applied).

By applying the values given in (19) and (20) to Eqs. (8), (17) and (18), the values of R as it increased in the light (in the absence of O_2) and decreased in the dark (in the presence and absence of O_2) were computed, and the results were compared with experimental data in Fig. 14. As may be seen, the agreement between calculated and observed values may be regarded as satisfactory.

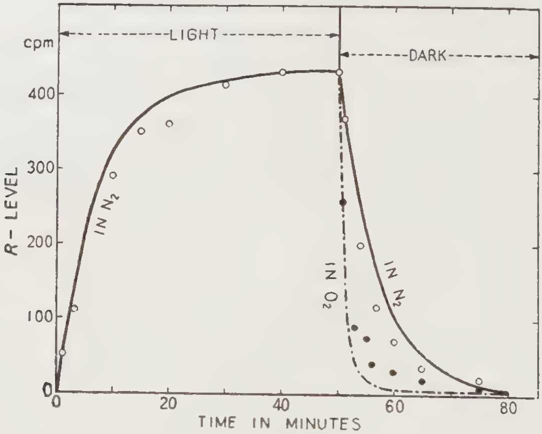
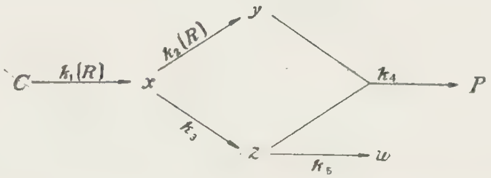


FIG. 14. Agreement between calculated and observed values of R as it increased in the light and decreased in the dark in the presence and absence of O_2. Explanation in text.

According to our notion, the inhibitory effect of O_2 on the normal photosynthesis is a consequence of the competition between C (in Eq. 14) and O_2 for R. The question arises, then, how we can interpret the fact that the suppression by O_2 of the R-level is of "first order" (Eqs. (2) and (12)), whereas the O_2-inhibition of normal photosynthesis is of "second order" (Eq. (1)). This apparent discordance may be reconciled by the following consideration. It should, first of all, be pointed out that in the mechanism of photosynthesis CO_2 is reduced to HCHO not by a single step as it is apparent from the simplest formula of the overall reaction: $CO_2+4H \rightarrow HCHO+H_2O$. If all the reductive steps involved are accomplished by participation of R, and if 2 atoms each of H are supplied by one molecule of R, we have to infer that R must participate in two stages in the mechanism of photosynthesis. As a simplest possibility, we may picture the photosynthesis to occur according to the following schema:



In this schema, C represents CO_2 or the primary carboxylation product derived from CO_2, P the ultimate photosynthate, x, y and z intermediate products, and w a certain byproduct which may be either oxidized away by respiration or in some way utilized again in the mechanism of photosynthesis. The rate constant of each reaction step is indicated on each arrow. As indicated, the reactions $C \rightarrow x$ and $x \rightarrow y$ proceed with the participation of the substance R. Besides these reaction steps, we assume the photochemical formation of R according to Reaction (3), and the back reaction of R (in the presence or absence of O_2) according to Reactions (4) and (5). The concentration of R will, therefore, change according to

$$\frac{d[R]}{dt}=k_{\mathrm{i}}[Q]-k'[R]-k''[O_2][R]-k_1[C][R]-k_2[x][R]$$

The concentration of R under stationary conditions is given by

$$[R]=\frac{k_{\mathrm{i}}[Q]}{k'+k''[O_2]+k_1[C]+k_2[x]}$$

We may assume, for the sake of simplicity, that under stationary conditions the concentration of the intermediate product x is very small, so that in the above equation the term $k_2[x]$ is negligibly small compared with other terms in the denominator. If this assumption is justified, the above equation may be written as

$$[R]=\frac{k_{\mathrm{i}}[Q]}{k'+k'']O_2]+k_1[C]} \tag{21}$$

Under stationary conditions, the rate of photosynthesis—which corresponds to $k_4[y][z]$—is given by

$$v=\frac{k_1k_2[R]^2[C]}{k_3+k_2[R]}$$

Substituting herein Eq. (21), we obtain

$$v=\frac{k_1k_2k_i^2[C][Q]^2}{(k'+k''[\mathrm{O_2}]+k_1[C])(k'k_3+k''k_3[\mathrm{O_2}]+k_1k_3[C]+k_2k_i[Q])} \tag{22}$$

In the absence of oxygen:

$$v_{\mathrm{N}}=\frac{k_1k_2k_i^2[C][Q]^2}{(k'+k_1[C])(k'k_3+k_1k_3[C]+k_2k_i[Q])} \tag{23}$$

According to these relations, the "degree of inhibition" caused by $\mathrm{O_2}$, which is defined by $H=1-\frac{v}{v_{\mathrm{N}}}$, is formulated as follows:

$$H=\frac{q[\mathrm{O_2}]+r[\mathrm{O_2}]^2}{p+q[\mathrm{O_2}]+r[\mathrm{O_2}]^2} \tag{24}$$

where

$$p=(k'+k_1[C])(k'k_3+k_1k_3[C]+k_2k_i[Q])$$
$$q=k''(2k'k_3+2k_1k_3[C]+k_2k_i[Q])$$
$$r=k''^2k_3$$

If q is reasonably small compared with $r[\mathrm{O_2}]$ *i.e.*, if

$$2k'k_3+2k_1k_3[C]+k_2k_i[Q]\ll k''k_3[\mathrm{O_2}],$$

Eq. (24) will assume the form:

$$H=\frac{[\mathrm{O_2}]^2}{\Phi^2+[\mathrm{O_2}]^2} \tag{25}$$

where

$$\Phi=\sqrt{\frac{(k'+k_1[C])(k'k_3+k_1k_3[C]+k_2k_i[Q])}{k_3k''^2}} \tag{26}$$

Eq. (25) is the same as that obtained experimentally (Eq. (1)), showing that oxygen causes a "second-order" inhibition of photosynthesis. The term $[C]$ in Eq. (26) may be thought to increase with increase of $\mathrm{CO_2}$ concentration. This equation, therefore, indicates that, in accordance with the observation made by Tamiya *et al.* (*4*), the value Φ will increase with the increase of $\mathrm{CO_2}$ concentration.

SUMMARY

1. The effect of molecular oxygen upon the $C^{14}O_2$-fixing capacity of *Chlorella* cells was investigated under various experimental conditions.

2. It was observed that the stationary level of the light-induced $C^{14}O_2$-fixing power (R) was markedly depressed by the effect of oxygen. The following relation was found to hold between the stationary R-level (established in the light) and the O_2-tension applied:

$$[R]_S = \left(\frac{\phi}{\phi + [O_2]} \right) [R]_S^N$$

where $[R]_S$ and $[R]_S^N$ represent the level established in the presence and absence, respectively, of oxygen, $[O_2]$ the oxygen partial pressure, and ϕ a constant which was found to be 0.22 atm. at 25°.

3. The depressing effect of oxygen upon the stationary R-level was increased when the concentration of $C^{14}O_2$ (supplied in the dark) was decreased.

4. The stationary R-level depressed by the effect of oxygen could be restored completely by the removal of oxygen.

5. While the process of formation (in the light) of the light-induced $C^{14}O_2$-fixing power was not affected by oxygen, its decay in the dark was markedly accelerated by the effect of oxygen. It was postulated that the level of the light-induced $C^{14}O_2$-fixing power is determined by the relative rates of formation (caused by light) and decay (occurring independently of light) of a certain reducing ($C^{14}O_2$-fixing) substance. Based on this view, a kinetic schema was presented to account for the various facts observed in the experiments. The essential conclusion arrived at was that both the inhibitory action of oxygen upon photosynthesis and the reactivity of oxygen as an oxidant for the Hill reaction are due to the reaction of oxygen with the reducing agent (R) formed by the effect of illumination.

6. In contrast to the light-induced $C^{14}O_2$-fixing power, the light-independent $C^{14}O_2$-fixing capacity of algal cells was found to be increased by the effect of oxygen.

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A PHOSPHORYLATIVE PROCESS, ACCOMPANIED BY PHOTOCHEMICAL LIBERATION OF OXYGEN, OCCURRING AT THE STAGE OF NUCLEAR DIVISION IN CHLORELLA CELLS. I.

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The technique of "synchronous culture" of *Chlorella*, which has recently been developed in our laboratory (*1*, *2*, *3*), has opened a new avenue of approach to the study of physiological and biochemical processes occurring in the life cycle of algal cells. It has been found that the cells of *Chlorella* assume, in the course of their growth, two distinct forms prossessing widely different characteristics. The form, which we called "dark cells", is smaller in size, richer in chlorophyll, and more photosynthetically active than the other form which was referred to as "light cells". The dark cells, when provided with light and adequate nutrient substances, grow into the light cells which in turn, under aerobic conditions, divide into a number of dark cells. The increase of nuclear substances occurs at a certain stage of light cells, the number of daughter (dark) cells to be produced from light cells being determined by the amount of nuclear substance (desoxypentose-nucleic acid) formed at the *ripening stage* of light cells (*3*). The measurement of photosynthetic gas exchange has revealed that the ripening stage of light cells is characterized by an enormously large value (2.0—4.0) of the apparent photosynthetic quotient (O_2/CO_2) (*2*). Using $C^{14}O_2$ as a tracer it was confirmed that, at the stage in question, the light-induced CO_2-fixing power of cells was decreased to a considerable degree (*2*). As a natural consequence of this fact the increase of cell mass ceased temporarily at the same stage. All these observations point to the occurence of a profound formative change at the ripening stage of light cells, and undoubtedly, the change is, as a whole, a reductive process as it is evidenced by the evolution of oxygen in excess of CO_2-absorption.

The work that is reported below was undertaken in an attempt to get more information as to the nature of the biochemical process occurring at the ripening stage of light cells. It was observed that the process is accompanied by an intake of considerable amount of inorganic phosphate which was found to be accumulated in cells, most probably, in the form of metaphosphate.

MATERIAL AND METHODS

Using *Chlorella ellipsoidea* as material, light and dark cells were prepared according to the method described in detail in our previous paper* (*1*). Cells were separated from the culture medium and were washed in three changes of distilled water by repeated centrifugation. When used in manometric experiments the cells were suspended in either of the following solutions:

(1) *M*/20 KH_2PO_4 solution (pH 4.5), for the experiments of phosphate-provision.
(2) *M*/40 tartrate buffer solution** (pH 4.6), for the experiments without provision of phosphate.

Absorption or evolution of O_2 and CO_2 were measured separately by Warburg's "indirect method" using two vessels, 25 ml. in volume, in which were filled 16 ml. and 10 ml., respectively, of the algal suspension containing 6.5—8.8 cu.mm. (packed

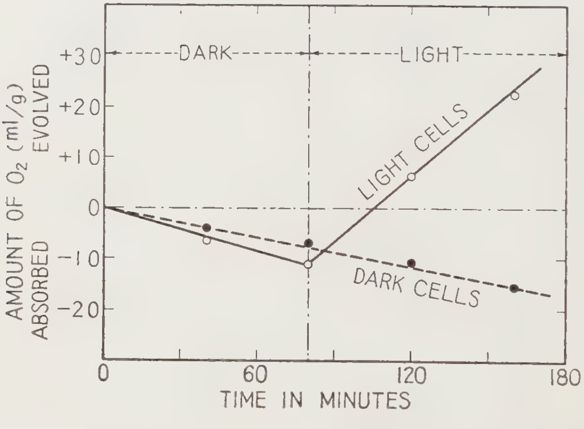

FIG. 1. The light-induced O_2-evolution by light cells in the presence of phosphate and absence of CO_2. Dark cells did not show the same phenomenon.

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* The culture solution used was of the following composition (per liter): KNO_3, 5.0 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; KH_2PO_4, 1.25 g; $FeSO_4 \cdot 7H_2O$, 0.003 g; Arnon's A5 solution (*4*), 1 ml.

** Tartrate buffer was found to have no injurious effect either on photosynthesis or on respiration of *Chlorella* cells.

volume) each of algae. Gas space of vessels was filled either with air containing 5 per cent CO_2, or, in the CO_2-free experiments, with air, in which case 0.5 ml. of 15 per cent KOH-solution was placed in the center well. The vessels were illuminated from below by tungsten lamps, the light intensity at the bottom of vessels being about 15–20 kilolux. The vessels were shaken with a rotary motion in a circle about 6 mm in diameter and at about 200–250 r.p.m. All experiments were performed at 25°.

The methods for analysing the phosphate compounds in algal cells will be described under the individual experiments.

RESULTS AND DISCUSSION

Light-induced O_2-evolution by Light Cells in the Presence of Phosphate

A marked difference between light cells (of the ripening stage) and dark cells was observed when their behaviors in the dark and light were compared in the presence of phosphate and absence of CO_2. In the experiment reproduced in Fig. 1, the cells, provided with phosphate and deprived of CO_2, were first kept dark and later illuminated. While the dark cells absorbed, both in the dark and light, oxygen at the same rate, light cells showed a remarkable gas-evolution on illumination. The evolved gas was confirmed to be oxygen by the fact that it was completely absorbed when NaHS or yellow phosphorus was placed in the side-bulb of Warburg's vessel. It should be noticed that this O_2-evolution occurred quite independently of the fixation of CO_2.

Using light cells of the ripening stage, the gas exchange occurring under illumination and in the presence of phosphate was followed with and without provision of CO_2. In Fig. 2, the rate of gas exchange (O_2-evolution and CO_2-absorption) are plotted against the time of experiment. As may be seen, the light-induced O_2-evolution in the absence of CO_2, which lasted more than two hours after the beginning of experiment, eventually ceased almost completely, and at the same time the rate of CO_2-absorption in the presence of CO_2 increased distinctly. Noteworthy is the fact that during the whole course of experiment the rate of O_2-evolution in the presence of CO_2 and phosphate remained quite unchanged, and, what is interesting, the difference between O_2-evolution and CO_2-absorption in the presence of CO_2 was almost equal to the O_2-evolution in the absence of CO_2. This fact seems to indicate that the O_2-production caused by phosphate occurred "competitively" with the O_2-evolution that was accompanied by the fixation of CO_2.

In Fig. 2 is also shown the time course of change of the apparent

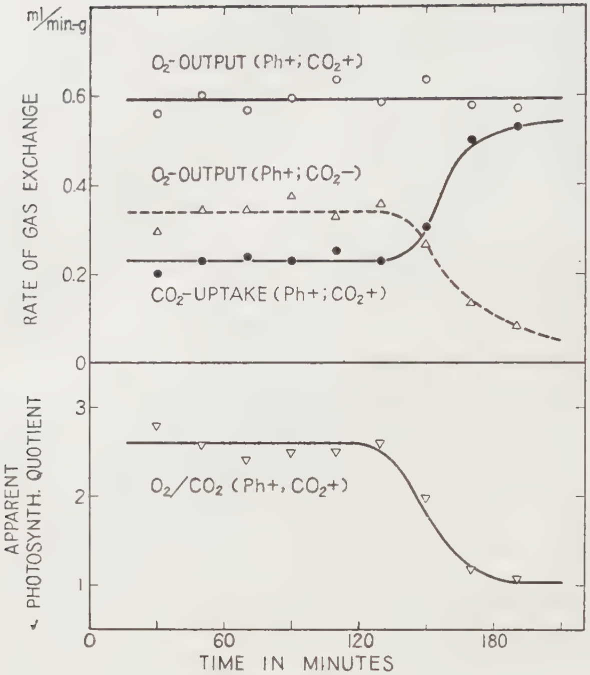

FIG. 2. Rate of gas exchange and the apparent photosynthetic quotient shown by illuminated light cells (suspended in phosphate solution) as it changed during the ripening process in the presence and absence of CO_2. Ph+ means the presence of phosphate, and CO_2+ and CO_2− the presence and absence, respectively, of carbon dioxide.

photosynthetic quotient (O_2/CO_2) observed in the presence of CO_2 and phosphate. In conformity with the observation reported in a previous paper (*2*), the quotient was high above unity at the ripening stage, but sank to the value around unity when the ripening was completed. It was actually observed, that the cells, soon after restoring the normal O_2/CO_2-quotient of unity, began to divide into daughter dark cells.

Significance of Phosphate for the Light-induced, CO_2-independent, O_2-evolution Shown by the Light Cells at the Ripening Stage

The process of light-induced, CO_2-independent, O_2-evolution shown by light cells was found to be markedly dependent on the existence of phosphate in the medium. In the experiment shown in Fig. 3, the light cells at the ripening stage were suspended, on the one hand, in phosphate solution, and on the other, in phosphate-free tartarate buffer, and under deprival of CO_2, their gas exchange (evolution or absorption of O_2) was compared with and without illumination. As is apparent from the figure, the positive O_2-evolution occurred only in the presence of phosphate. When deprived of phosphate, the cells showed, even in the light, an absorption of oxygen, which proceeded at a rate that was almost equal to the rate of respiration (in the dark) in the presence of phosphate.

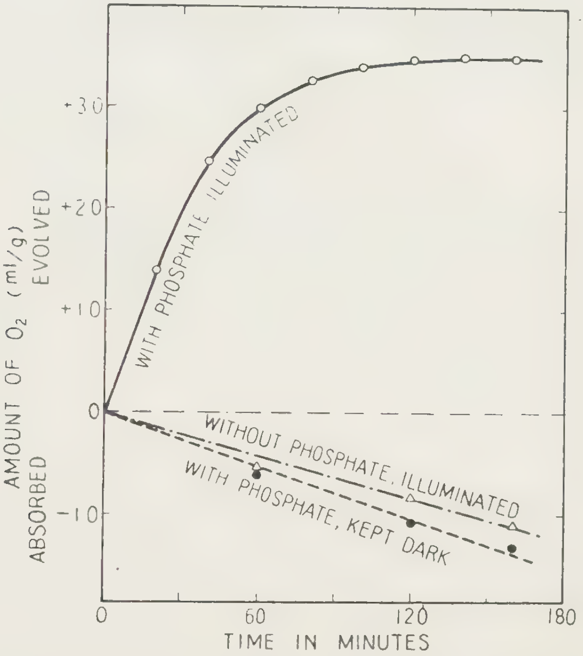

FIG. 3. Effect of phosphate on the process of light-induced, CO_2-independent, O_2-evolution shown by light cells. All experiments were performed under deprival of CO_2.

An interesting phenomenon was observed when the light cells, under provision of CO_2, were illuminated in the presence and absence of phosphate. From the data presented in Fig. 4, it may be seen that the process of O_2-evolution itself proceeded at the same rate in phosphate and tartarate buffers. In the absence of phosphate, CO_2-absorption occurred at the same rate as O_2-evolution, with a result that the photosynthetic quotient was unity. When phosphate was present, the CO_2-absorption was distinctly suppressed so that the apparent photosynthetic quotient became high above unity. This result shows clearly that the photochemical O_2-evolution in the presence of CO_2 occurs at a definite rate without being affected by the presence of phosphate, whereas the process of CO_2-absorption is markedly suppressed by the effect of phosphate.

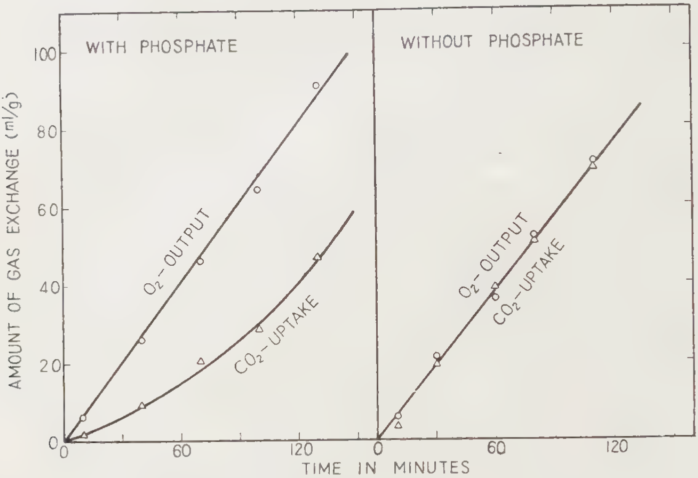

FIG. 4. Effect of phosphate on the gas exchange (O_2-output and CO_2-uptake) shown by light cells under illumination and in the presence of CO_2. While O_2-output was not affected by phosphate, CO_2-uptake was distinctly lowered in the presence of phosphate.

Using the light cells actively showing the phenomenon in question, an experiment was made to ascertain whether the phosphate was incorporated in algal cells during its action of provoking the light-induced O_2-evolution. The cells were suspended in a phosphate solution, and under deprival of CO_2 they were incubated for 1.5—2.5 hours in the light and dark, and the phosphate content of algal cells before and after the incubation was measured by Allen's method (*5*).* It was found that, while the dark-incubated cells did not assimilate phosphate, the light-incubated light cells accumulated a considerable amount of phosphate during the incubation. Such an accumulation of phosphate did not occur either in dark cells or in the light cells which had completed their sporulating process showing the decrease of apparent photosynthetic quotient to unity (*cf.* Fig. 2).

TABLE I

Quantitative Relation between O_2-evolution and Phosphate-Assimilation Shown by the Light Cells which Were Incubated for 1.5–2.5 Hours in the Light under Deprival of CO_2

O_2 evolved per mg. of algae		P assimilated per mg. of algae		$\frac{O_2 \text{ evolved in moles}}{P \text{ assimilated in moles}}$
μl.	*μM*	*μl.*	*μM*	
16.2	0.72	6.0	0.19	3.8
10.0	0.45	4.0	0.13	3.5
32.2	1.43	13.1	0.42	3.4
20.6	0.92	9.6	0.31	3.0
				Av.: 3.4

Using 3–10 mg. (dry weight) of light cells of the ripening stage, quantitative measurements were made of the O_2-evolution and phosphate-fixation which occurred during 1.5–2.5 hours' incubation in the light under deprival of CO_2. The results obtained are summarized in Table I, from which it may be seen that the molar ratio of O_2 evolved/phosphate assimilated was 3.4 in average.

Metaphosphate as the Possible Main Product of Light-induced Phosphate-fixation in Light Cells

To make clear the question in what form the assimilated phosphate exists in light cells, the cells were suspended (under deprival of CO_2) in solutions of ordinary phosphate or P^{32}-containing phosphate, and after incubation for 2 hours in the light or dark, the cells were analysed for their content of phosphorus compounds in various fractions.* The fractions separated were as follows:

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* The cells were washed three times with distilled water before being analysed.

(1) "Acid-soluble fraction" which was obtained by extracting cells for 15 minutes with 5 per cent trichloroacetic acid at room temperature.

(2) "Alcohol-ether-soluble fraction" which was obtained by extracting the residue of (1) with 3 : 1 alcohol-ether mixture for 20 minutes at room temperature.

(3) "Acid-insoluble fraction" which was obtained from the residue of (2) by heating for 5 minutes at 100° with 5 per cent trichloroacetic acid.

(4) "Residue" which represents the remains of (3).

The results obtained are summarized in Tables II and III. From Table II it may be seen that about 75–90 per cent of the phosphate taken up during incubation in the light were recovered in the "acid-insoluble fraction"*. The per cent of recovery in the same fraction was not so large when P^{32} was applied (Table III). This situation may be attributed to the occurence of exchange of P^{32} with P in all phosphorus compounds, as it is evidenced by the fact that even the unilluminated cells showed fairly high contents of P^{32} in various fractions.

TABLE II

Distribution of P in Various Fractions Obtained from Light Cells before and after Incubation (for 2 Hours) in the Light

P was determined by Allen's method after incineration of samples in the presence of perchloric acid.

	P in mg. per g. (dry weight) of algal cells		
	Before incubation	After incubation in the light	Increase caused by illumination
Acid-soluble fraction	1.7	2.6	0.9
Alcohol-ether-soluble fraction	1.6	2.6	0.5
Acid-insoluble fraction	8.0	13.0	5.0
Residue	2.9	2.9	0
Sum	14.2	20.6	6.4
Total P observed	13.9	20.2	6.3

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* When the cells were incubated in the dark with the provision of ordinary phosphate, the amount and distribution of P remained unchanged, at least in so far as the incubation lasted for 2 hours.

Nevertheless the ratio of P^{32} found in illuminated and non-illuminated cells was the greatest in the "acid-insoluble fraction" as may be seen from the figures given in Table III.*

TABLE III

Distribution of P^{32} in Various Fractions Obtained from the Light Cells which Were Incubated (for 2 Hours) in the Light or Dark with Provision of Radioactive Phosphate

	Count per minute and per mg. (dry weight) of algal cells which have been		
	Incubated in the dark (D)	Incubated in the light (L)	L/D
Acid-soluble fraction	339	851	2.5
Alcohol-ether-soluble fraction	48	134	2.8
Acid-insoluble fraction	67	337	5.0
Residue	32	74	2.3
Sum	486	1396	2.9
Total P observed	495	1450	2.9

Attempt was made to follow out more in detail the nature of the compound accumulated in the "acid-insoluble fraction". The following tests were made using the acid-insoluble fraction obtained in the experiment given in Table II.

(i) The sample was directly treated with ammonium molybdate to determine the inorganic phosphate (P_i).

(ii) Inorganic phosphate was precipitated and removed by treating the sample with 10 per cent $CaCl_2$ solution saturated with $Ca(OH)_2$ at pH 8.8.

(iii) The sample which has been freed from inorganic phosphate was subjected to:

(a) 7 minutes' hydrolysis with 1 N HCl and the phosphorus liberated as orthophosphate was determined (P_7),

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* When dark cells were incubated in the presence of P^{32}, the P^{32}-uptake owing to the exchange reaction was found to be more or less accelerated by the effect of illumination, although the ratio of P^{32} in illuminated and non-illuminated cells remained the same in all fractions.

(b) treatment with barium acetate at pH 3.5–4.0 and the precipitated phosphorus (P_b) was determined,

(c) treatment with chitosan (poly-glucosamin) at pH 4.0–7.0 (after removal of trichloroacetic acid from the sample), and the precipitated phosphorus (P_c) was determined.

TABLE IV

Nature of the Phosphorus Compound in the "Acid-insoluble Fraction" Obtained from the Light Cells which Were Incubated (for 2 Hours) in the Light

P_i: inorganic phosphate; P_7: the phosphorus which appeared as orthophosphate after 7 minutes' hydrolysis with 1 *N* HCl; P_b: the phosphorus which was precipitated by barium acetate at pH 3.5–4.0; P_c: bound phosphorus which was precipitated by chitosan at pH 4.0–7.0.

	P in mg. per g. (dry weight) of algal cells		
	Before incubation	After incubation in the light	Increase caused by illumination
Total Acid-soluble fraction	8.0	13.5	5.0
P_i	1.0	2.8	1.8
P_7	2.5	5.5	3.0
P_b	2.0	5.1	3.1
P_c	2.3	5.3	3.0

The results obtained are presented in Table IV. From the figures given in the fourth column of the table it may be seen that practically all of the phosphorus accumulated by the effect of illumination consists of inorganic phosphate (*ca.* 36 per cent) and the phosphorus of the compound (*ca.* 60 per cent) that was hydrolyzable by 7 minutes' treatment with 1 *N* HCl. The latter compound, as may be seen from the data in the table, is 100 per cent precipitable with barium acetate and chitosan, a fact which suggests strongly that the compound in question is metaphosphate, which is known to be completely hydrolyzed by 7 minutes' treatment with 1 *N* HCl (*6*). To make a further check on the nature of the compound, the sample precipitated by barium acetate was treated with toluidine blue which is known to give a characteristic metachromatic reaction in the presence of metaphosphate (*7*). The reaction was found to be distinctly positive, indicating that the substance is most probably metaphosphate.

It should be remarked that, according to Yoshida (*8*, *9*), metaphosphate is hydrolyzed to about 30 per cent when heated with 5 per cent trichloroacetic acid for 5 minutes (see also Wiame (*6*)). In our experiment this procedure was taken for separating the "acid-insoluble fraction". The fact that about 36 per cent of the increased phosphorus found in the "acid-insoluble fraction" was inorganic orthophosphate may be due to the occurrence of hydrolysis of metaphosphate during this procedure of fractionation.

SUMMARY

1. Using the technique recently devised by Tamiya *et al.* (*1*), *Chlorella ellipsoidea* was cultured "synchronously", and the biochemical process occurring in the life cycle of alga was investigated with special reference to the stage of nuclear division ("ripening") of wholly grown up cells or the "light cells" according to the terminology of Tamiya *et al.*

2. It was found that, when the "light cells" of the ripening stage were illuminated in the presence of phosphate, they consumed a considerable amount of phosphate with simultaneous evolution of oxygen. The photosynthetic CO_2-absorption was more or less suppressed when phosphate was present and assimilated by cells with the simultaneous evolution of O_2. As a consequence, the light cells at the ripening stage showed an apparent photosynthetic quotient (O_2/CO_2) high above unity in the presence of phosphate, whereas they showed normal photosynthetic quotient ($O_2/CO_2 \doteqdot 1$) in the absence of phosphate and in the presence of CO_2. In short, the phosphate in this case functions, outwardly, in the manner of the Hill reagent, causing, in competition with CO_2, a photochemical reduction of some cellular material.

3. In the absence of CO_2, absorption of 1 mole of phosphate was accompanied by the evolution of about 3.4 moles of O_2.

4. The phosphorus fixed photochemically by the ripening light cells was found, in the main, in the fraction which was obtained by extraction of cell materials for 5 minutes with 5 per cent trichloroacetic acid at 100°, after the cells were extracted with cold 5 per cent trichloroacetic acid and alcohol-ether (3 : 1) mixture. The phosphorus compound contained in this fraction was hydrolyzable by 7 minutes' treatment with 1 *N* HCl, precipitable with barium acetate at pH 3.5–

4.0 and with chitosan at pH 4–7, and showed a distinct metachromatic reaction with toluidine blue. These properties suggest strongly that the major portion of the phosphate fixed was in the form of metaphosphate.

This work was carried out as a part of the program directed by Professor Hiroshi Tamiya, and it is a pleasure to the author to express his gratitude for his expert guidance. Thanks are due to the Ministry of Education and the Rockefeller Foundation for grants in aid of this work.

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BIOCHEMICAL STUDIES ON PATHOGENIC FUNGI
III. ON THE PROTEINASE OF TRICHOPHYTON GYPSEUM

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Pathogenic fungi have not attracted the attention of medical biochemists until recently. Unlike the other microorganisms such as bacteria and viruses, even fundamental studies on the organisms are scarce. However, the recent discoveries of many antibiotics which are dramatically effective against various bacterial infections, have stimulated search for further undiscovered antibiotics or chemotherapeutics against pathogenic fungi. At the same time biochemical studies in this field have advanced because, as can be easily understood, fundamental studies on a microorganism are of great help directly or indirectly in the discovery and improvement of therapeutic agents against infection caused by the specific organism.

In view of the above, we undertook extensive biochemical investigation of pathogenic filamentous fungi, particularly those belonging to the dermatophytes which are parasitic on man and animals, attacking the superficial keratinized areas of the body such as skin, hair and nails. We have already reported (*1*) the findings concerning the chemical composition of *Trichophyton gypseum* which is one of several species most frequently isolated from human trichophytosis in our country, and the present paper is a part of enzymatic studies on this fungus.

As to the proteolytic activities of fungi, those for non-pathogenic fungi have been reported by a number of workers, and an outline of the information available up to around 1950 has been described by Ito (*2*). Recently Dion (*3*, *4*) and McConnel (*5*) investigated the distribution and nature of the extracellular proteolytic enzymes of 289 species of fungi and actinomycetes. However, we could find only a few reports available regarding pathogenic fungi including dermatophytes. Kamibayashi (*6*) earlier studied the distribution of proteinase, together with carbohydrases and lipases, among 14 species of dermatophytes and reported the existence of proteolytic activity in all the organisms tested, though he did not examine the nature of the proteinase action in detail.

From the preliminary experiments described in the preceding paper of this series (*7*), we obtained some basic information on the proteolytic activities of *Trichophyton gypseum*. This paper deals with the nature of the proteinase in this fungus.

EXPERIMENTAL

Test Organism—A strain of *Trichophyton gypseum* which had been provided by Prof. Yamamoto of Kyoto University was used throughout the experiments. The organism was cultivated on Sabouraud's 2 per cent glucose broth at 25° and the mycelial pad formed was harvested after a definite period, usually two weeks.

Enzyme Preparation—The mycelial pad, after being washed repeatedly with physiological saline solution, was ground in a mortar with double its weight of glycerol-water mixture (1 : 1) and filtered through gauze. The maceration thus obtained was used as an enzyme source. Sometimes a water extract of acetone powder of the mycelium was also used. The extract was prepared as follows; the ground mycelium was treated with three volumes of acetone, centrifuged after stirring, and the operation further repeated twice and the powder finally obtained was then treated with ether, filtered and dried over sulfuric acid in a vacuum desiccator after evaporation of the ether. The 3 per cent suspension of the acetone powder was left standing at 0° for 24 hours and centrifuged at 4,000 r.p.m. for 30 minutes, and the supernatant fluid was used for the experiments.

Cell-free supernatant fluids of maceration, acetone powder extract and of culture medium prepared by centrifugation at 12,000 r.p.m. for 30 minutes, were also used as enzyme sources.

Test Procedure—Mixture of the enzyme, substrate and buffer solution of appropriate pH totalling 20 ml. was incubated at 38° under toluene. As substrates, casein, gelatin and peptone (Polypeptone, Takeda Pharm. Co.) were generally used. McIlvaine's citrate buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid) was exclusively used throughout the experiments. Control solution containing enzyme, buffer and the same amount of water instead of substrate solution, was treated in exactly the same manner. After a definite time, the acidity increase in 4 ml. of the digestate was estimated by the formol titration with 0.05 N NaOH solution. The values obtained from the control experiments were deducted from those of the main experiments, and the corrected values are shown in the tables.

RESULTS AND CONSIDESATIONS

Optimal pH for Proteinase and Peptonase—As is seen from Table I, casein, gelatin and peptone are hydrolyzed both in acid and alkaline reactions, but all substrates did not undergo cleavage at a strong acid reaction, namely below pH 4 to 5, and the optimal pH for hydrolysis was similar to that of trypsin and lay all around pH 8.2 after 4 hours' incubation. The optimum for the three substrates agreed very closely. It was also found that the optimal pH did not change appreciably depending upon the age of culture of the organism, namely each enzyme preparation from 2, 4, and 8 week cultures alike had the optimal zone around pH 8.2.

Table I

Optimal pH for the Proteinase Activity

Reaction mixture: 5 ml. 4% substrate solution+5 ml. maceration of 2 week culture+10 ml. buffer solution; incubated at 38° under toluene.

		Acidity increase in 4 ml. digestate (ml. 0.05 *N* NaOH)										
Substrate	pH Time	4.0	5.0	6.0	7.0	7.6	7.8	8.0	8.2	8.4	8.6	9.0
Casein (Hammersten)	*hrs.* 4	0	0.15	0.18	0.43	0.76	0.83	0.86	**0.98**	**0.94**	0.87	0.82
	24	0	0.52	0.73	1.53	1.99	2.01	2.04	**2.09**	**2.08**	2.05	1.88
	72	0	0.88	2.09	2.74	2.91	2.95	2.93	**3.03**	**3.00**	2.95	2.75
Gelatin	4	0	0.12	0.13	0.26	0.39	0.38	**0.39**	**0.38**	0.37	0.35	0.31
	24	0	0.22	0.29	0.68	0.82	0.81	**0.91**	**0.96**	0.87	0.87	0.84
	72	0	0.27	0.73	0.35	1.57	1.54	**1.81**	**1.80**	1.63	1.65	1.53
Peptone (Polypeptone)	4	0	0	0.14	0.16	0.15	0.24	**0.30**	**0.31**	0.20	0.16	0.13
	24	0	0	0.30	0.28	0.37	0.52	0.55	**0.63**	0.50	0.39	0.30
	72	0	0	0.39	0.40	0.48	0.54	0.58	**0.63**	**0.69**	0.49	0.47

Hydrolysis Grade of Various Protein Substrates—As shown in Table II, all of the proteinaceous substrates tested were split to a considerable degree, except for fibrin which underwent slight cleavage only after a long period of incubation. Generally speaking, casein and protamine were digested most rapidly and the relative rate of hydrolysis diminished in the following order: Protamine > casein > gelatin > edestin > egg-albumin > peptone > fibrin.

Influence of Proteinase Inhibitors and Activators—Recently it has been proposed to classify the proteolytic enzymes into three general groups based on the conditions required for the manifestation of their activity (*8*). The first group includes the enzymes which require no activator,

Table II

Hydrosis Grade of Various Proteins

Reaction mixture: 5 ml. 4% substrate solution+3 ml. maceration of 2 week culture+12 ml. buffer solution; incubated at pH 8.0, 38° under toluene.

	Acidity increase in 4 ml. digestate (ml. 0.05 *N* NaOH)		
Substrate \ Time *hrs.*	24	72	120
Protamine	0.87	3.69	3.69
Casein	1.78	2.73	3.62
Gelatin	1.59	1.60	2.33
Edestin	0.70	1.74	2.18
Egg-albumin	0.70	1.58	1.78
Fibrin	0.00	0.33	0.61
Peptone (Polypeptone)	0.62	0.89	1.25

while those belonging to the second group are activated by such substances as cyanide, cysteine, hydrogen sulfide and so on, and the last group by various heavy metal ions. The proteinase of the first group can be further divided into tryptase, pepsin type enzyme, *etc.* as judged by the optimal pH for their respective action and the behaviour against enzyme inhibitors. Therefore, in the present investigation, the effects of proteinase inhibitors and activators as well as the optimal pH of the enzyme activity were carefully studied in an attempt to classify the proteinase in question.

As egg-white (*9*), serum (*10*) and soybean (*11*, *12*) have been known to contain a powerful trypsin inhibitor, the effects of these substances upon the *Trichophyton* proteinase were examined. As shown in Table III, human serum and hen's egg-white in fifty times dilution exhibited an inhibitory effect upon gelatinase activity, though the former was almost without effect upon the crude enzyme preparations such as maceration and acetone powder extract. As to the trypsin inhibitor in soybean, it has been known to inhibit many proteinases found in fungi, though Pechmann (*13*) reported the resistance of his *Aspergillus* proteinase against this inhibitor. Gelatinase of the dermatophyte under investigation was found to be susceptible to the crude trypsin inhibitor from soybean prepared according to Ham and Sandstedt (*12*). The results and the method of preparation of the inhibitor are presented in Table IV.

TABLE III

Inhibitory Actions of Human Serum and Hen's Egg-white

Reaction mixture: 4 ml. 5% gelatin solution+4 ml. enzyme preparation +4 ml. inhibitor solution in 50 times dilution+8 ml. buffer solution; incubated at pH 8.0, 38° under toluene.

The values of the control experiments without inhibitor were subtracted from those of the main experiments with inhibitor, and these corrected values are given in parentheses in the table.

		Acidity increase in 4 ml. digestate (ml. 0.05 *N* NaOH)		
Enzyme preparation	Inhibitor / Time (*hrs.*)	Serum	Egg-white	None (Control)
Maceration	24	0.97 (−0.04)	0.55 (−0.46)	1.01
	48	1.24 (−0.06)	0.78 (−0.52)	1.30
Cell-free supernatant fluid of maceration	24	0.11 (−0.61)	0.34 (−0.38)	0.72
	48	0.20 (−0.64)	0.45 (−0.39)	0.84
Acetone powder extract	24	0.64 (−0.01)	0.36 (−0.30)	0.66
	48	0.69 (−0.07)	0.44 (−0.32)	0.76
Cell-free supernatant fluid of the extract	24	0.10 (−0.30)	0.15 (−0.25)	0.40
	48	0.15 (−0.53)	0.35 (−0.33)	0.68

The effects of the additions of cysteine, hydrogen sulfide, cyanide and heavy metal ions such as magnesium, manganese, zinc, cobalt and iron, upon proteinase activity were also examined. But these substances showed no activating effect upon the proteinase examined.

Thus, it was revealed that the proteinase of *Trichophyton gypseum* was not activated by the various protease activators tested, whereas it was susceptible to three kinds of trypsin inhitors. These results as well as information on the optimal pH would indicate the tryptase nature of the proteinase of this fungus. It must be cited here tha the majority of the proteinases found in bacteria and in fungi have been reported to have trypsin-like characteristics. Our results agree with these observations.

TABLE IV

Inhibitory Effect of Soybean Trypsin Inhibitor

Reaction mixture: 4 ml. 5% gelatin solution+4 ml. maceration of 2 week culture+4 ml. inhibitor solution+8 ml. buffer solution; incubated at pH 8.0, 38° under toluene.

Preparation of the inhibitor solution: Ten grams of dried soybean were macerated with 50 ml. water and the solution was adjusted to pH 4.2 with *N* sulfuric acid. The supernatant fluid of this maceration was mostly deproteinized by precipitation with kaolin. The inhibitor was further purified by precipitation of the supernatant fluid with acetone added to 60% final concentration, and the resulting precipitate was extracted with 10 ml. water.

The values of the control experiments without inhibitor were subtracted from those of the main experiments with inhibitor, and these corrected values are given in parentheses in the table.

Acidity increase in 4 ml. digestate (ml. 0.05 *N* NaOH)		
Time	Inhibitor added	No addition (Control)
hrs. 24	0.47 (−0.60)	1.07
48	0.73 (−0.98)	1.71

Proteinase Action of Various Enzyme Preparations—Four kinds of the enzyme preparations were tested as shown in Table V. The activity was still demonstrable in aqueous extract of the dried mycelium which

TABLE V

Proteinase Activities of Various Enzyme Preparations

Reaction mixture: 5 ml. 4% gelatin solution+5 ml. enzyme preparation +10 ml. buffer solution; incubated at pH 8.0, 38° under toluene.

Acidity increase in 4 ml. digestate (ml. 0.05 *N* NaOH)			
Time *hrs.* / Preparation	24	48	72
Maceration	1.35	1.49	2.50
Cell-free supernatant of maceration	0.83	0.86	1.13
Acetone powder extract	0.95	1.28	1.72
Cell-free supernatant of the extract	0.49	0.69	1.07

had been prepared as described above. Furthermore, the enzyme could be easily separated free from fungus cells by centrifugation at 12,000 r.p.m. for 30 minutes.

Influence of Culture Conditions upon the Formation of Proteinase—Influence of culture time upon the production of the enzyme and distribution of the activity between fungus mycelium and culture medium were investigated. The results obtained are presented in Table VI. The proteinase activity of the mycelium did not change appreciably after cultivation for about one week. It is interesting to notice that the medium of young culture contained little activity while the mycelial pad of the same culture had already aquired strong activity, although the activity in the medium increased slightly in the older culture. These findings would more reasonably be explained as being due to the fact that the proteinase of the fungus is originally intracellular in nature and the little activity found in the culture fluid is to be attributed to the liberation of the enzyme by autolysis of the fungus cells.

TABLE VI

Influence of Culture Time upon the Production of Proteinase

Reaction mixture: 5 ml. 4% casein solution + 5 ml. enzyme preparation + 10 ml. buffer solution; incubated at pH 8.0, 38° under toluene.

Acidity increase in 4 ml. digestate (ml. 0.05 *N* NaOH)			
Culture time	Preparation / Time	Mycelium maceration	Culture-medium filtrate*
weeks	*hrs.*		
1	24	3.24	0.18
	72	4.20	0.24
3	24	3.10	0.63
	72	4.03	1.33
8	24	2.20	0.66
	72	3.40	1.34

* Filtered through Seitz bacterial filter.

Influence of the composition of the culture medium was also studied. The fungus was cultivated on a synthetic fluid medium containing no proteinaceous material, the composition of which was shown in Table VII. Three grams of the mycelial pad thus formed was ground to make a maceration in the same way as mentioned above, and was proved to contain proteolytic activity upon gelatin. The average value of 0.05 *N* NaOH in ml. of duplicate experiments, corresponding to acidity increase in 4 ml. digestate after 24 hours' incubation (reaction mixture: 5 ml. 4 per cent gelatin solution+3 ml. maceration+12 ml. buffer solution; incubated at pH 8.0, 38° under toluene), was 0.61. The fact that the synthetic medium which contained no protein substrates also gave rise to the production of the proteinase would indicate the non-adaptive nature of the enzyme formation by the organism.

TABLE VII

Composition of Synthetic Medium

Glucose	20.0 g.
Alanine	5.0
NH_4NO_3	5.0
Na_2HPO_4	1.0
$NaHCO_3$	1.0
KCl	1.0
$CaCl_2$	0.01
$Mg(NO_3)_2$	0.001
$ZnSO_4$	0.001
$MnSO_4$	0.001
$FeSO_4$	0.001
$Co(NO_3)_2$	0.001
Thiamine	0.01
Distilled water	to 1,000 ml.
pH adjusted	to 6.0

SUMMARY

The nature of proteinase activity of *Trichophyton gypseum* was investigated and the following results were obtained.

1. The maceration of the mycelium hydrolyzed casein, gelatin and peptone considerably in pH range of 5 to 9, and the optimal pH for hydrolysis was found to be at pH 8.2.

2. The degree of hydrolysis of various proteins at pH 8.0 diminished in the following order: Protamin > casein > gelatin > edestin > egg-albumin > peptone > fibrin.

3. Proteinase activity was inhibited by egg-white, human serum and soybean trypsin inhibitor, but not affected by cysteine, cyanide, hydrogen sulfide or heavy metal ions.

4. The proteinase activity was also found in the extract of acetone powder of the mycelial pad, and the enzyme was easily extractable with water, free from fungus cells.

5. The enzyme seemed to be constantly (not adaptively) produced by the organism and only slightly liberated into the culture fluid.

From these facts the proteinase of *Trichophyton gypseum* was considered to belong to the category of tryptase.

This work was carried out as a part of a comprehensive investigation of the pathogenic fungi under the direction of Prof. Yuki Ito, M.D., to whom the author is indebted for his constant guidance.

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ON THE MECHANISM OF THE ACTIVATION OF MOLECULAR OXYGEN BY HEMOGLOBIN

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On the reaction of autoxidation of oxyhemoglobin (HbO_2) and also on the fact that hemoglobin can act as an oxidase under certain conditions, many investigations have hitherto been made and various theories on their reaction mechanisms have been presented (*1–8*). In this paper, the reactions of hemiglobin formation from HbO_2 under various conditions were more profoundly investigated and a new scheme of the reaction mechanism of the hemiglobin formation from HbO_2 was hypothetically presented.

EXPERIMENTAL

Crystalline equine oxyhemoglobin prepared by Heidelberger's method which was improved in some points by Suzuki *et al.* (*9*), was dissolved in 0.1 *M* KCl as the stock solution. The sample was proved to be free from catalase. The methods were used to follow up the reaction of hemiglobin formation as follows; i) determination of O_2 output by Warburg's manometric technique, ii) determination of hemiglobin by photometric method with the spectrophotometer of Hitachi Co. The actual reading of the optical density was carried out with hemiglobin in the forms of HiCN or HiN_3 by the addition of KCN or NaN_3 to the reaction solution. One definite isosbestic point was found in the plots of absorption figures of HbO_2, HiCN, HiN_3, namely at 565 mμ. Further, the ratio of $\varepsilon_{578}/\varepsilon_{565}$ for each of HbO_2, HiCN and HiN_3 was determined empirically to be 1.61, 0.78 and 1.0, respectively. By adopting these values, two kinds of nomogram were established each representing the relation between $\varepsilon_{578}/\varepsilon_{565}$ and the relative composition in percentage of HbO_2 and HiCN or HiN_3 in the solution. Using this nomogram, the amounts of hemiglobin formed at each time of the reaction could be determined readily by the ratio of $\varepsilon_{578}/\varepsilon_{565}$ found.

RESULTS

I. Autoxidation of Oxyhemoglobin

Effect of pH—Autoxidation of oxyhemoglobin was accelerated by decrease of pH (Fig. 1). The hemiglobin formation proceeded as a first order reaction at least in earlier reaction stages. The relationship between the reaction velocity and pH is shown in Fig. 2. The velocity of O_2 evolution from HbO_2 was also proved to increase with decrease of pH. (Fig. 3). The maximum O_2 liberation was found in the reaction at pH 5.15. Theoretically, the maximum volume of O_2 liberated from HbO_2 should be expected to amount as much as 75 per cent of the total O_2 of HbO_2 unless any of the reducing radicals other than fer-

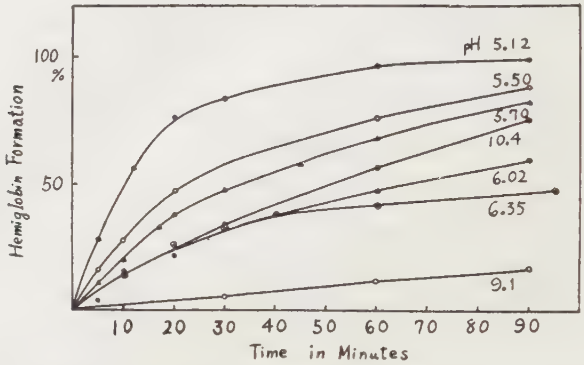


FIG. 1. Autoxidation of HbO_2 at various pH. (photometric measurements)
HbO_2 (as heme): $9.3\times10^{-5}\,M$, KCl: $8.8\times10^{-2}\,M$
Buffer: acidic side, $1.8\times10^{-1}\,M$ acetate buffer; alkaline side, $1.9\times10^{-1}\,M$ carbonate buffer
T: 37°

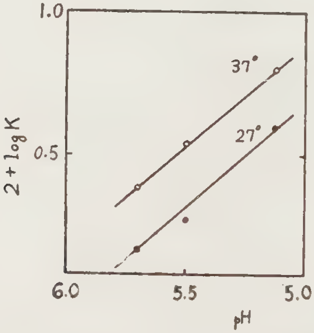


FIG. 2. Relationship between the rate of HbO_2 autoxidation and pH.

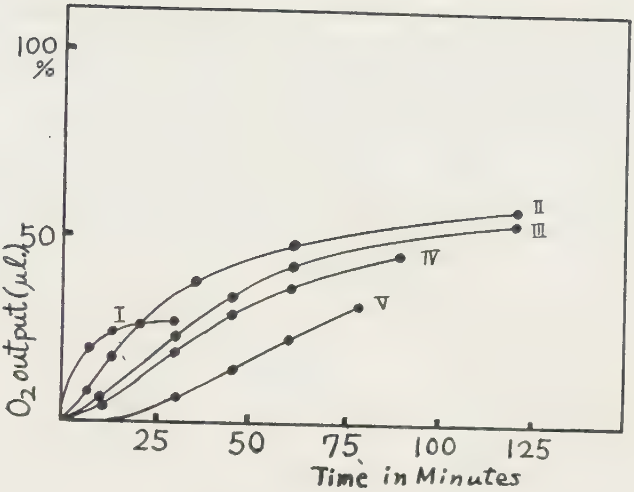


FIG. 3. Autoxidation of HbO_2 at various pH. (manometric measurements)
pH: I. 4.4; II. 5.15; III. 5.5; IV. 5.9; V. 6.8
T: 37°

rous iron of hemoglobin is oxidized by oxygen. The maximum volume of liberated O_2 actually found was always lower than that theoretically expected, though it reached usually over 50 per cent of the total O_2 of HbO_2. It may be presumable by this fact that some reducing groups of the protein part of hemoglobin molecule would certainly be oxidized by molecular oxygen in the course of the autoxidation. The amounts of oxygen consumed by the protein part of hemoglobin molecule at each pH condition were calculated and presented in Table I. The amount of oxygen consumed per mole of hemoglobin was surprisingly large; it amounted to as much 1.54 moles even at pH 5.15.

The results shown in Fig. 3 was represented in Fig. 4 as the plots of log $(a-x)$ against time, where a was taken as 75 per cent of the total oxygen of HbO_2. The O_2 evolution proceeded essentially as a first order reaction at every pH. The reaction rate became smaller, however, after longer reaction time, so that a break was noticed on the time curve of the reaction. Similar relation was noticed also in the reaction followed up spectrophotometrically. Any explanation on these facts should be reserved at present.

TABLE I

The Amounts of Oxygen Consumed by the Protein Part of Hemoglobin Molecule at Each pH Condition

pH	O_2 consumed per mole HbO_2	Hemiglobin formed	Reaction time
	moles	%	*min.*
4.4	2.8	100	30
5.15	1.54	97	145
5.5	1.90	80	120
5.9	2.13	58	90

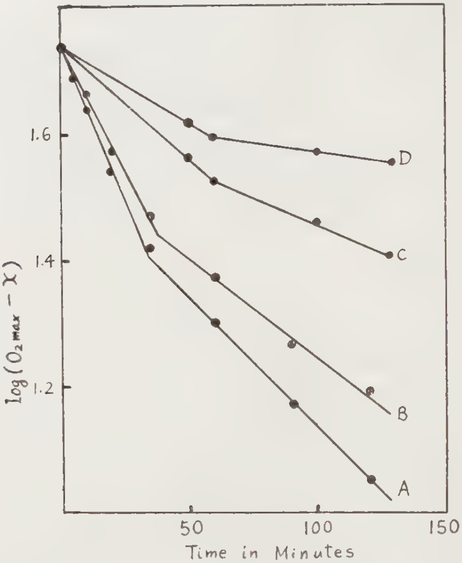


FIG. 4. Logarithmic representation of the reaction of HbO_2 autoxidation at various pH. (derived from Fig. 3)
pH: A, 4.4; B, 5.15; C, 5.5; D, 5.9

Effect of the Perturbators—As was reported by Tsushima (*10*) in our laboratory, the autoxidation of oxyhemoglobin was accelerated on addition of some sort of perturbators such as sodium benzoate or salicylate. In connection with the above observation, the reaction of autoxidation in the presence of sodium benzoate was now followed up manometrically. The time course of the O_2 evolution in the present reaction system is shown in Fig. 5, a. In some of the experiments, reaction solution was taken out from the manometer vessel at a definite time of reaction and the amount of hemiglobin formed was measured spectrophotometrically. If no O_2 was consumed by the protein part of the hemoglobin, the theoretical O_2 evolution calculated should be those shown by Fig. 5, b. Accordingly, the curve c (difference of curve a and b) in Fig. 5 may represent the amount of O_2 consumed by the oxidation of the protein part of hemoglobin molecule. The values of O_2 consumed as such in each reaction with varied concentrations of benzoate were calculated by the same procedure and are shown in Table II. Within the benzoate concentration lower than 1 *M*, the O_2 uptake was about 1.8 moles per molecule of hemoglobin. With ben-

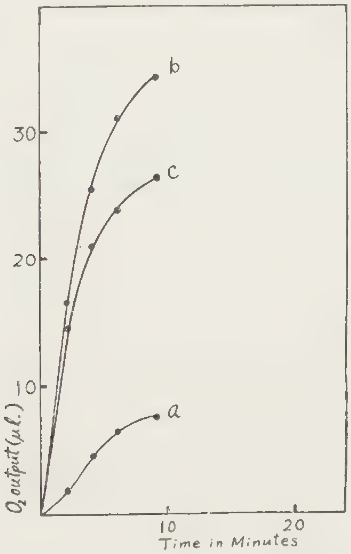


FIG. 5. Effect of Sod. Benzoate upon the autoxidation of HbO_2.

a: O_2 output actually found
b: O_2 output theoretically calculated
c: b—a
T: 30°

TABLE II

The Amounts of Oxygen Consumed by the Protein Part of Hemoglobin Molecule at Each Sodium Benzoate Concentration.

T: 30°. pH 7.5

Concentration of sodium benzoate	O_2 consumed per mole HbO_2	
M	*mole*	
0.8	1.4	(3)
1.0	1.87	(5)
1.25	2.32	(8)
1.5	2.32	(7)
1.75	2.28	(8)
2.0	2.21	(5)

The presented values of O_2 consumed per mole HbO_2 are the average values of the several experiments. Numbers of these determinations are given in the brackets.

zoate concentrations above 1 *M*, however, O_2 uptake was found to be as high as 2.4 moles.

Effect of Catalase—As seen from Figs. 6 and 7, the rate of HbO_2 autoxidation was supressed, though slightly, by the addition of catalase. The fact may suggest that some peroxides would have been formed in the system and played a role in the reaction of HbO_2 autoxidation.

Structural Changes of Hemoglobin Molecule—The ordinary product of HbO_2 autoxidation is hemiglobin. At the pH lower than 5.0, however, the absorption curve of the reaction product was not always in agreement with that of acid hemiglobin; it approached gradually to that of hemichrome as the pH was lowered. In the reactions at the pH higher than 10, the absorption figure of the reaction product resembled that of alkali hematin. In all of the cases, however, the absorption figure of the reaction solution after the treatment with NaOH and $Na_2S_2O_4$ agreed completely with that of protohemochrome. Accordingly, no disintagration of heme molecule should have been occurred in the course of HbO_2 autoxidation.

II. Oxidation of HbO_2 by Potassium Ferricyanide

Effect of pH—As is well known, when HbO_2 was oxidized to hemi-

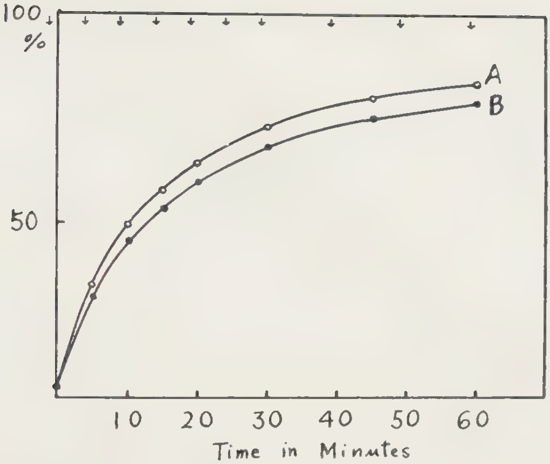


FIG. 6. Effect of catalase on the autoxidation of HbO_2. (photometric measurements)

A: control
B; with catalase added
0.1 ml. of 6.9×10^{-6} *M* catalase was added at each allow
pH: 4.9 T: 37° HbO_2 (as heme): 9.2×10^{-5} *M*

globin by ferricyanide, the O_2 evolution corresponds to the total O_2 of HbO_2. The rate of O_2 evolution, however, was influenced significantly by pH as shown in Fig. 8. With respect to the reaction of hemiglobin formation from HbO_2 by ferricyanide, there are many evidences to believe that it is not HbO_2 but reduced Hb which is actually oxidized by ferricyanide (*11*, *12*). Betke (*12*), for instance, showed that reduced Hb prepared by evacuation was oxidized by ferricyanide much faster than HbO_2. On the other hand, O_2 affinity of Hb decreases with lowering of pH ("Bohr effect"). Our findings shown in Fig. 8 can reasonably be explained by these considerations.

Effect of Perturbators—The rate of oxidation of HbO_2 by ferricyanide was accelerated also by the addition of perturbators such as sodium laurate or benzoate. The results of the experiments with sodium laurate are shown in Fig. 9. The concentration of the perturbators effective for this acceleration was found to be very low. The highest acceleration of hemiglobin formation was realized by laurate at the concentraiton of 1 mole of laurate per 1 mole of heme. These facts may suggest that the configuration of the protein part of hemoglobin

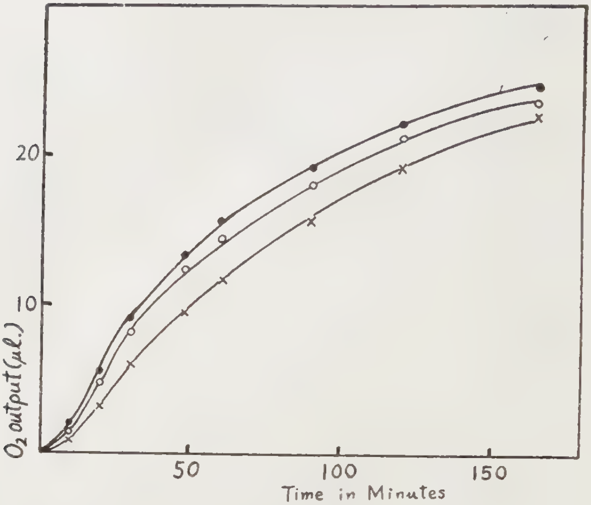


FIG. 7. Effect of catalase on the autoxidation of HbO_2. (manometric measurements)

HbO_2 (as heme): $0.35\times10^{-3}\,M$ T: 30° pH: [illegible]5.2

-×-: control, without the presence of catalase

-○-: in the presence of $2.4\times10^{-9}\,M$ catalase

-●-: in the presence of $2.4\times10^{-7}\,M$ catalase

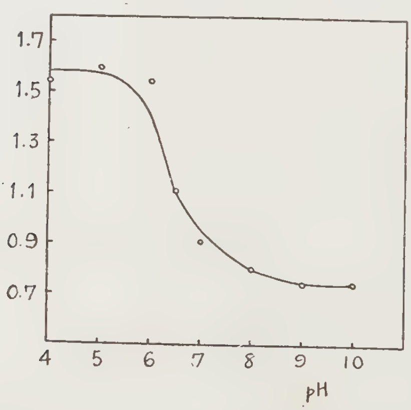


FIG. 8. Relationship between the rate of the oxidation of HbO_2 by Ferricyanide and pH.

Ordinate: reciprocal value of the time in minutes for the half oxidation of HbO_2.

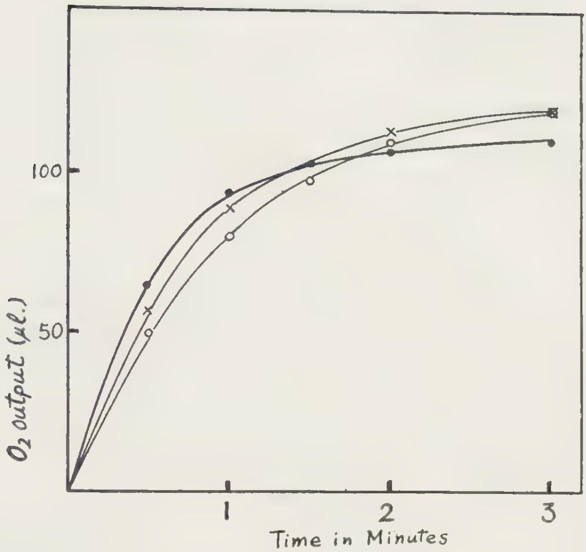


FIG. 9. Effect of Sod. Laurate upon the oxidation of HbO_2 by Ferricyanide.

HbO_2 (as heme): 1.12×10^{-3} *M*
Ferricyanide: 4.48×10^{-3} *M*
Laurate: -○-, 0 *M*
-●-, 4.48×10^{-3} *M*
-×-, 4.48×10^{-4} *M*

molecule might be modified already by such a low concentration of laurate.

III. Hemiglobin Formation from HbO_2 in the Presence of Hydrogen Donor

Effect of Potassium Ferrocyanide—In the study of the effect of hydrogen donor on the hemiglobin formation from HbO_2, it is necessary to use adequate reductants which are inert against the reduction of hemiglobin once formed. It has been pointed out by Schuler (*13*) that hemiglobin can be reduced, though slightly, by ferrocyanide. In the present experiments, however, the concentration of ferrocyanide was very low, so that the reduction of hemiglobin by ferrocyanide may be practically neglected. Serving as a hydrogen donor, ferrocyanide will be oxidized to ferricyanide which in turn will be reduced again to ferrocyanide through the oxidation of HbO_2 to hemiglobin; ferrocyanide may thus act as a catalyst in the present reaction of hemiglobin formation.

The results of the experiments with ferrocyanide are shown in Figs. 10 and 11. The rate of hemiglobin formation was greatly promoted by the addition of ferrocyanide. The curves in Fig. 11 representing the O_2 evolution during the reaction, are of a sigmoidal character especially in their early stages.

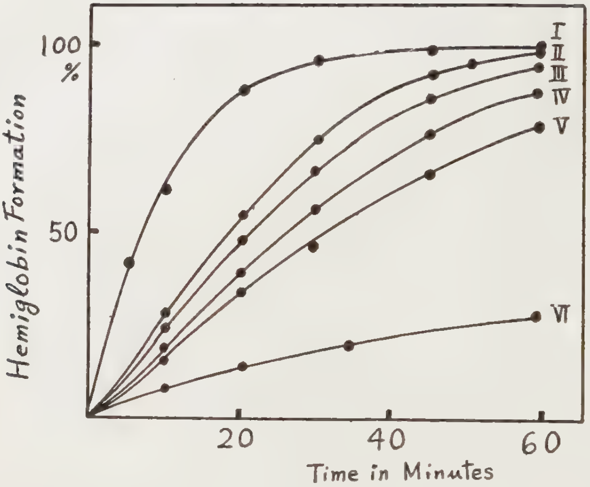


FIG. 10. Effect of Ferrocyanide upon the reaction of hemiglobin formation from HbO_2. (photometric measurements)
HbO_2 (as heme): $5\times10^{-5}\,M$ pH: 6.5 T: 37°
Ferrocyanide: I. $2\times10^{-4}\,M$; II. $1\times10^{-4}\,M$; III. $5\times10^{-5}\,M$; IV. $2.5\times10^{-5}\,M$; V. $1.25\times10^{-5}\,M$; VI. $0\,M$

Effect of Sodium Azide—NaN_3 was found to be a very suitable hydrogen donor for the present experiments. Azide does not reduce hemiglobin, further it combines with hemiglobin to form HiN_3 as soon as hemiglobin is formed and the catalytic activity of hemiglobin can thus be completely inhibited. The amount of hemiglobin formed, therefore, can easily and immediately be measured spectrophotometrically as HiN_3. The results of the experiments obtained by spectrophotometry are shown in Fig. 12. The same but rather qualitative observations have been formerly made also by Sjöstrand (7). The reaction rate increased with increase of the concentration of azide

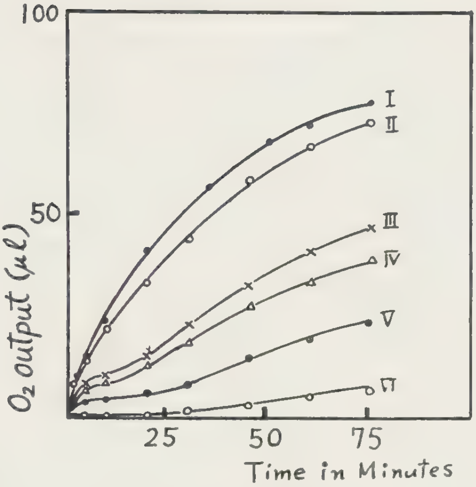


FIG. 11. Effect of Ferrocyanide upon the reaction of hemiglobin formation from HbO_2. (manometric measurements)

HbO_2 (as heme): $1.3 \times 10^{-3}\,M$ T: 37°

Ferrocyanide: I. $5.2 \times 10^{-3}\,M$; II. $2.6 \times 10^{-3}\,M$; III. $1.3 \times 10^{-3}\,M$; IV. $0.65 \times 10^{-3}\,M$; V. $0.325 \times 10^{-3}\,M$; VI. $0\,M$

added. The rate of gas evolution also increased significantly as shown in Fig. 13. But, the total amount of the gas evolved was found to be greater than that expectable as total O_2 of HbO_2. Presumably other gases such as N_2O and NO might be evolving in addition to O_2. As a matter of fact, we have found by titrating N_3 with $AgNO_3$, that considerable amounts of NaN_3 were consumed in this reaction. In addition, as seen from Fig. 13, O_2 absorbtion was observed in the early stage of the reaction, especially when the concentration of NaN_3 used was relatively low.

Effect of the Further Addition of Ascorbic Acid in the Presence of NaN_3—Ascorbic acid is one of the most popular hydrogen donor. As, however, ascorbic acid reduces hemiglobin readily, it is difficult to investigate directly the effect of ascorbic acid on the reaction of hemiglobin formation. When, however, a sufficient amount of NaN_3 has been added to the reaction solution previously, all the hemiglobin would be trapped as HiN_3 as soon as hemiglobin was formed and as ascorbic acid does not reduce HiN_3 into reduced Hb, it is possible to investigate selectively the effect of ascorbic acid on the reaction of hemiglobin

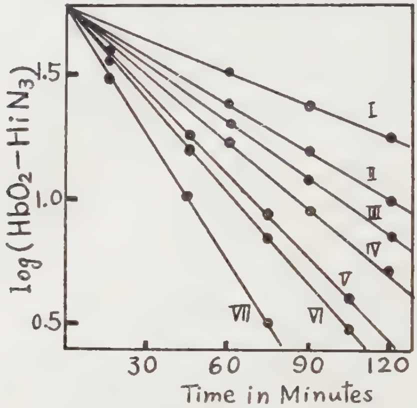


FIG. 12. Effect of NaN_3 upon the reaction of hemiglobin formation from HbO_2. (photometric measurements)
HbO_2 (as heme): $0.5\times10^{-4}\,M$ pH: 6.0 T: 37°
NaN_3: I. $0\,M$; II. $0.5\times10^{-4}\,M$; III. $1\times10^{-4}\,M$; IV. $2.5\times10^{-4}\,M$; V. $5\times10^{-4}\,M$; VI. $1\times10^{-3}\,M$; VII. $2.5\times10^{-2}\,M$

formation. The experimental results showing the effect of ascorbic acid are presented in Fig. 14. The rate of hemiglobin formation was increased as the increasing amount of ascorbic acid was added.

DISCUSSION

Considering the above results of experiments, the reaction mechanism of HbO_2 autoxidation may be expressed as follows:

$$(1)\quad HbFe^{II}O_2 \rightleftarrows [HbFe^{II}O_2^*] \rightleftarrows HbFe^{II}+O_2$$

$$\quad\quad \longrightarrow HbFe^{III}+\cdot O_2 \xrightarrow{H_2O} HbFe^{III}OH^- + HO_2 \quad \cdots(1)$$

$$(2)\quad HbFe^{II}O_2+HO_2 \xrightarrow{H_2O} HbFe^{III}OH^- + H_2O_2 + O_2 \cdots\cdots(2)$$

$$(3)\quad HbFe^{II}O_2+H_2O_2 \longrightarrow HbFe^{III}OH^- + HO + O_2 \cdots\cdots(3)$$

$$(4)\quad HbFe^{II}O_2+HO \longrightarrow HbFe^{III}OH^- + O_2 \cdots\cdots(4)$$

$$4HbFe^{II}O_2 \longrightarrow 4HbFe^{III}OH^- + 3O_2$$

In this reaction scheme, an activated state of HbO_2 designated as $HbFe^{II}O_2^*$ was postulated as an intermediate between $HbFe^{II}O_2$ and $HbFe^{II}$. Thus, the first stage of $HbFe^{II}$ oxidation by molecular oxygen takes place in the state of $HbFe^{III}O_2^*$. As the first step of the autoxidation of HbO_2, $HbFe^{III}$ and $\cdot O_2$ would be formed as shown by Eq. (1).

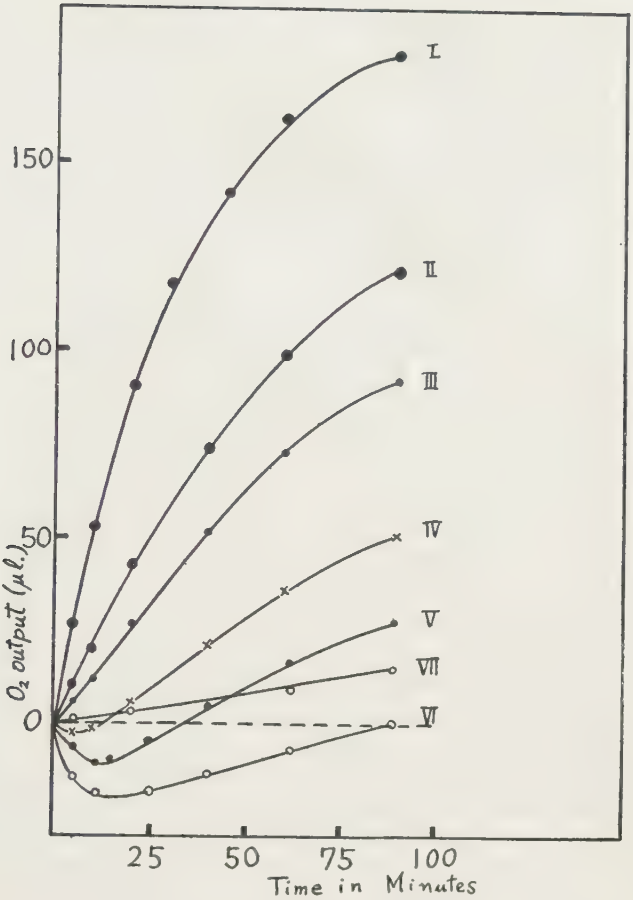


FIG. 13. Effect of NaN_3 upon the reaction of hemiglobin formation from HbO_2. (manometric measurements)

HbO_2 (as heme): $1.39 \times 10^{-3}\,M$. pH: 6.4 T: 37°.

NaN_3 in $10^{-2}\,M$: I. 4.1; II. 1.25; III. 0.625; IV. 0.25; V. 0.125; VI. 0.625; VII. 0

Then $\cdot O_2$ would be converted into HO_2. The next step of the reaction is that which leads to the liberation of free O_2 as indicated by Eq. (2) followed by further reaction chain of O_2 liberation. To sum up the reaction steps 1 to 4, 75 per cent of the total oxygen of HbO_2 should be liberated theoretically. When catalase is added into the reaction

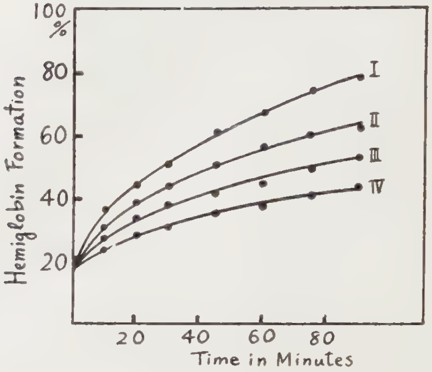
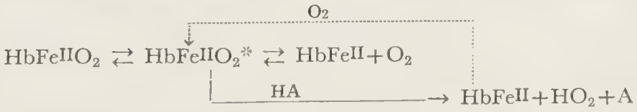


FIG. 14. Effect of ascorbic acid upon the reaction of hemiglobin formation from HbO_2 in the presence of NaN_3.
HbO_2 (as heme): 0.25×10^{-4} *M*
NaN_3: 0.25×10^{-3} *M* pH: 6.0 T: 18°
Ascorbic acid: I. 0.25×10^{-2} *M*; II. 1×10^{-3} *M*; III. 0.5×10^{-3} *M*; IV. 0

solution, H_2O_2 or some free radicals of the same kind would in part be decomposed and as a result of this, the reaction rate of autoxidation should be decreased. As these peroxide and the related free radicals are much more active as oxidant than free O_2, they may easily oxidize the reducible groups of the protein part of the hemoglobin molecule. The fact that the amount of O_2 evolution experimentally found was lower than the theoretical value of 75 per cent may plausibly be explained by the above assumption. In addition to it, the final sum of O_2 evolved in the HbO_2 autoxidation was found in the presence of catalase O_2 to be usually larger than that without catalase and also this fact may certainly be in responce to the catalatic decomposition of H_2O_2 or related free radicals.

Acccording to the above scheme of reaction mechanism, the reaction rate of autoxidation may fundamentally depend upon the reaction in which $HbFe^{II}$ is oxidized by O_2 in the state of $HbFe^{II}O_2^*$. And after that the $\cdot O_2$ was once produced, the reaction rate of hemiglobin formation would depend upon the reaction between $HbFe^{II}O_2$ and HO_2, H_2O_2 or HO. And as it has been assumed that it is not HbO_2 but reduced Hb which is actually oxidized by the oxidants, the probability of the hemiglobin formation by the action of H_2O_2 or related free radicals would also depend upon the amount of free $HbFe^{II}$ presenting in the reaction solution. These assumptions may also be supported by the expertmental facts reported by Brooks (*2*), George (*5*) and Sjöstrand (*7*), respectively, that the rate of the autoxidation of HbO_2 or oxymyoglobin increased with decreasing pO_2, although it decreased again on pO_2 below 20–25 mmHg. Now, as shown in Figs. 8 and 9, the rate of O_2 evolution in the reaction of HbO_2 oxidation by ferricyanide was significantly increased with decreasing pH or by the addition of a slight amount of perturbator. On the other hand, it has previously been reported by the authors (*14*) that in the reaction of choleglobin formation by the reaction system of Hb—ascorbic acid—O_2, the rate of both choleglobin formation and ascorbic acid oxidation were greatly increased by the addition of perturbators in such a slight amount that there occurred no visible degree of perturbation of hemoglobin molecule. The effective concentration range of the perturbators to accelerate the O_2 evolution in the reaction of HbO_2 oxidation by ferricyanide was proved to be nearly the same to that which was found as effective in the reaction of choleglobin formation. Certainly, by the addition of the perturbators in such low concentrations, the protein part of hemoglobin molecule might have been already modified in some way; the affinity of hemoglobin to O_2 has been found to be decreased as the consequence, followed by a striking increase of hemiglobin formation in the reaction of HbO_2 autoxidation and even of the decomposition of heme in the reaction of choleglobin formation. It may be assumed additionally that according to the modification of the protein part, also the function of heme of hemoglobin would be affected to some degree, entailing the increase of the oxidative activity of hemoglobin. Based on these considerations, the fact may plausibly be construed as indicating that HbO_2 autoxidation is accelerated also by the addition of perturbators as reported by Tsushima and again by the present authors.

Now, accepting the afore-mentioned mechanism of HbO_2 autoxidation, much greater HO_2 production might be expected when some proper hydrogen donor was added to the reaction solution, because these hydrogen donors would react more readily with the activated O_2 of $HbFe^{II}O_2^*$ and produce HO_2 according to the following scheme.



Thus the hemiglobin formation from HbO_2 should be accelerated.

The experimental results shown in Figs. 10, 11, 12 and 14 are in good accordance with the above assumption. In the presence of hydrogen donor, reduced Hb should be formed in the initial stage of the reaction which would return to HbO_2 by combining with O_2. Accordingly, the O_2 liberation may be expected to be smaller in the early stage of reaction and may eventually be an O_2 absorption. In fact, we were able to ascertain the O_2 evolution curve of the reaction with ferrocyanide to be essentially of a sigmoidal character. It was also proved that O_2 was actually absorbed in the early stage of the reaction in the presence of a slight amount of NaN_3. These facts may well support the reaction scheme presented above.

From all of the experimental results above described, it may be concluded in the first place that the rate of hemiglobin formation from HbO_2 by molecular oxygen is increased by the factors which are concerned with increasing production of HO_2, namely by some hydrogen donors present in the reaction solution. Not only the hydrogen donor added intentionally into the reaction solution, but the reducing radicals of hemoglobin molecule may also be effective as hydrogen donors. Secondly, the rate of hemiglobin formation can be increased by the factors which decrease the affinity of hemoglobin to O_2. Decreased pH and the addition of perturbators are included in this group of factors. Thirdly, the factors which would modify the function of heme of hemoglobin toward the increase of its oxidative activity may be considered. The factors in the third group may presumably be those which causes more or less modifications of the configuration in protein part of the hemoglobin molecule. It has been pointed out by many researchers (*4*,*6*) that when HbO_2 was strongly acidified by HCl or trichloracetic acid in the presence of some reductants such as ascorbic acid or bilirubin, the reductants will be instantly oxidized to a large extent by acid denaturation of HbO_2. It is obvious from these facts that O_2 is activated by hemoglobin at the moment of the denaturation of protein in hemoglobin molecule. Thus we may reasonably expect that the activity of hemoglobin as an oxidative catalyst can strikingly be increased by the configurational modification of the protein part. With respect to the effect of the perturbators on the reaction of both choleglobin formation and HbO_2 autoxidation, the explanation may be conformed on the same basis.

SUMMARY

With the aim to clarify the mechanism of the activation of molec-

ular oxygen by hemoglobin, the reaction of hemiglobin formation from HbO_2 under various conditions were studied both spectrophotometrically and manometrically. The main results obtained are as follows:

1. Autoxidation of HbO_2 is accelerated by lowered pH and by the addition of perturbators such as sodium benzoate and laurate. The affinity of hemoglobin to O_2 seems to decrease under these condition.
2. The rate of autoxidation of HbO_2 is decreased by the co-existence of catalase in the reaction solution. The fact suggests that some peroxides would have been formed in this system and played a role in this reaction.
3. No disintegration of heme molecule is proved in the reaction of HbO_2 autoxidation, but the protein part of hemoglobin molecule seems to be modified considerably.
4. The rate of oxidation of HbO_2 by ferricyanide is also significantly accelerated by lowered pH and by the addition of perturbators. This may suggest that it is not HbO_2 but reduced Hb which is actually oxidized by the oxidants.
5. The rate of hemoglobin formation is greatly promoted by the addition of some adequate reductants such as ferrocyanide, NaN_3, and ascorbic acid which would concern the increasing production of HO_2 in the reaction solution.
6. Considering the above facts, a hypothetical scheme of the reaction of hemiglobin formation in both the presence and absence of the reductants was presented. It should be pointed out especially that the activated state of HbO_2 designated as $HbFe^{II}O_2^*$ was introduced in the scheme as a presumption.
7. The possible significance of our demonstrations on the interrelation between the function of heme and the structural modification in the protein part of the hemoglobin molecule was discussed.

The authors wish to thank to Prof. K. Kaziro for his interest and useful discussions during the course of this work. This work were supported by the Science research Fund of the Ministry of Education for which the authors also with to thank.

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PROTEIN SYNTHESIS IN EXCISED SILKGLAND OF SILKWORM. I.*

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(Received for publication, February 8, 1955)

In recent years many investigators have employed isotopically labeled amino acids to study the process of protein synthesis. An incorporation of labeled amino acids into protein of tissue slices or homogenates, has been regarded as evidence of protein synthesis *in vitro*, but no net increase in quantity of protein has been confirmed.

Recently, however, direct observations of protein formation *in vitro* has been reported. Hokin (*1*) has described a method for measuring the formation and secretion of amylase by pigeon pancreas slices. Peters and Anfinsen (*2*, *3*) have demonstrated the net increase of serum albumin in liver slices by using a quantitative immunological technique. Gerarde, Jones and Winnick (*4*) have also observed that the cultivation of embryochick tissue in roller tubes, in the presence of an extract of chick embryo, resulted in an increase in protein content of the cultures.

In the present paper, a quantitative experiment was undertaken to study the synthesis of protein by silkgland of silkworm (*Bombyx mori*, L.) *in vitro*. Silkgland is an organ which is evolved to be specially adapted for synthesis of silk protein. Therefore, this gland seems to be a suitable material for studing the process of protein synthesis.

The incorporation of labeled glycine into protein of isolated silkgland was reported by Zamecnik *et al.* (*4*). Sisakyan and Kuvaeva (*5*) observed an increased synthesis of protein in the culture medium containing amino acids and body fluid of pupa of silkworm. In the experiment presented below, the tissue of silkgland was used and the amount of protein formed during incubation was directly determined.

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* A preliminary report on this work has already been published (*7*).

MATERIALS AND METHODS

Preparation and Incubation of Silkgland Tissue

Silkworms (*Bombyx mori*, L: a hybrid "Japanese 115 × Chinese 108"), in the 3rd to 5th day of the 5th instar, were dissected in a physiological solution, which contained 11.20 g. of NaCl, 0.24 g. of KCl, 0.14 g. of $MnCl_2$ and $MgCl_2$, 0.02 g. of $NaHCO_3$, 1.50 g. of $NaH_2PO_4 \cdot 2H_2O$ and 1.50 g. of glucose per liter, and was adjusted to pH 8.0 with 1N NaOH solution. A middle silkgland was cut off and its jellylike content (liquid silk) was removed from the gland tissue. The separation of viscous contents from the gland tissue was a delicate procedure. It was found that silkworm in the 3rd to 5th day of the 5th instar was most suitable for the preparation of tissue. A tube-form tissue, approximately 15 mm in length, was connected to a glass capillary of about 1 mm in diameter. The connection was made by adhering with cemedine (synthetic resin). The inner wall of the gland tissue was rinsed thoroughly with the physiological solution to remove the protein remaining on the gland wall. An injection tube was the most convenient apparatus to pour the washing solution into the cavity of gland tissue.

The gland tissue was then sealed with cemedine at another end and transfered to a culture vessel, which contained 5 ml. of culture medium. The tissue was incubated at 30°, as illustrated in Fig. 1. The composition of the basal culture medium is shown in Table I. The cavity of gland tissue was filled with the physiological solution upto the level higher than the surface of outer solution, in order to maintain the gland tissue bulgy. Care was taken not to injure the tissue, since even the presence of a slight injury resulted in a effluence of protein from the gland tissue cavity. From 20 to 30 minutes elapsed from the time of dissection until the culture vessels were placed in the bath.

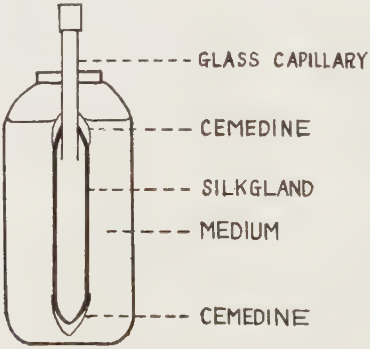


FIG. 1. Diagram of silk gland incubated.

Determination of Protein Formed.

At the end of each incubation period, the tissue was carefully taken out from the vessel. The contents of gland cavities were then transferred into small test-tubes (4 mm. × 65 mm.) by cutting the tip of gland tissue with a small pair of scissors. The inside of the gland was washed with small portions of the physiological solution. The combined solution did not exceed 0.2 ml. in volume. This solution was analyzed for protein.

The amount of protein formed was nephelometrically determined using "Sueyoshi's reagent" by the modification of Sueyoshi's method (*8*). The combined solution containing protein was made to 0.2 ml. with the physiological solution and then 0.2 ml. of Sueyoshi's reagent was added.

After standing for 12 to 15 hours at room temperature, the tubes were vigorously shaken. The turbidity of the solutions were then compared with a series of standard solutions, which were prepared by adding 0.2 ml. each of Sueyoshi's reagent to an equal volume of ovalbumin solution. The protein contents of the standard solutions ranged from 50 to 1000 γ per tube, with intervals of 50 γ. In this paper, the estimated amount of protein was expressed in terms of γ per silkgland.

The tests were repeated ten to twenty times, and the average value was shown in each tables and figures. The experimental error was found to be ±50 γ.

Basal Culture Medium

The compositions of the basal culture medium is shown in Table I. When the effects of various substances on the protein formation were studied, each substance was added to this basal culture medium, the pH being adjusted to 8.0 with 1 *N* NaOH solution before use.

In the earlier stage of this experiments, the effect of two culture media of different amino acids composition, which were similar to that of fibroin and sericin,

TABLE I

Composition of Basal Medium

Substance	Concentration	Substance	Concentration
NaCl	11.20 *g./l.*	Glycine	0.58 *g./l.*
KCl	0.24	DL-Alanine	1.16
$MnCl_2$	0.14	DL-Serine	0.58
$MgCl_2$	0.14	L-Leucine	0.58
$NaHCO_3$	0.02	L-Aspartic acid	0.58
$NaH_2PO_4 \cdot 2H_2O$	1.50	L-Na-Glutamate	0.58
Glucose	1.50	L-Arginine·HCl	0.58
		L-Tyrosine	0.34

respectively, were compared and the latter was found to give better results. Accordingly, in all experiments described here, the latter culture medium was employed.

RESULTS

Effect of pH—In Figs. 2 and 3 the effect of pH on protein formation by gland tissue is shown. The culture media were adjusted to the desired pH with 1 *N* HCl or 1 *N* NaOH. In no instance was a significant change in pH observed at the end of the incubation time. It seems that the reaction of protein formation is optimum between pH 7.8 to 8.5 with a peak at pH 8.0. It should be noted that the optimum pH for protein formation was considerably alkaline compared with the pH of the body fluid of silkworm, which was found to be from 6.3 to 6.8.

Time of Incubation—The effect of the time of incubation on protein formation by gland tissue is illustrated in Figs. 4 and 5. On incubating

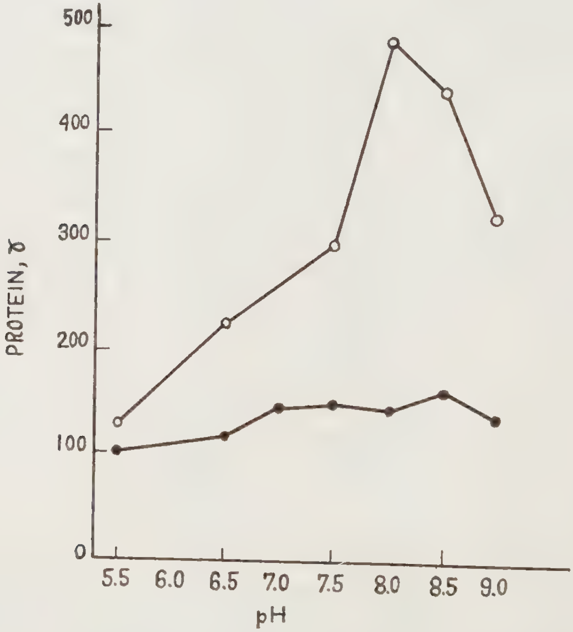
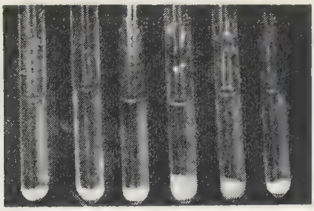


Fig. 2. Effect of pH on protein formation by silk gland.

○ Basal medium + 0.003 *M* pyruvate

● Basal medium



Basal medium+0.003 M pyruvate

FIG. 3. Effect of pH on protein formation

in a medium containing glucose and pyruvate, the amount of protein formed increased progressively for the first 2 hours, but no increase in protein was observed on further incubation. On the basis of this results, the gland tissues were incubated for 1 hour in most of the experiments

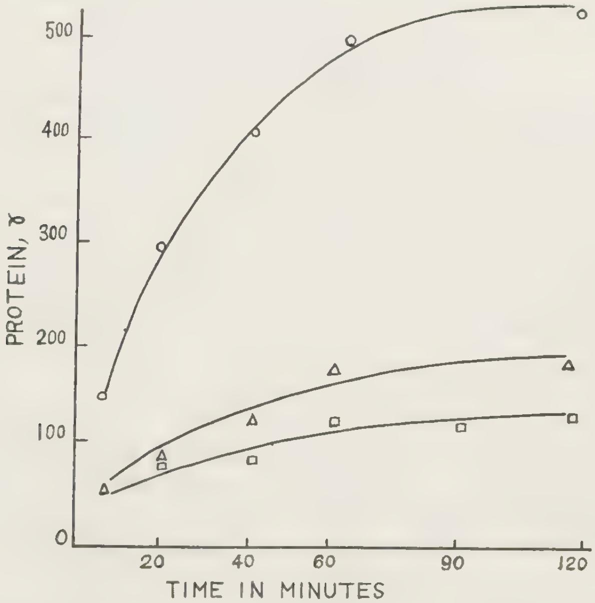


FIG. 4. Effect of time of incubation on protein formation

□ Basal medium
△ Physiological solution+0.003 M Pyruvate
○ Basal medium+0.003 M pyruvate

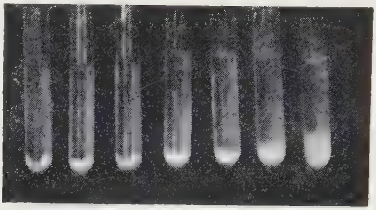


FIG. 5. Effect of time of incubation on protein formation

represented in this paper.

Effect of Phosphate—Table II shows the effect of phosphate on the protein formation by gland tissue. When phosphate was omitted from the culture medium, a remarkable decrease in protein formation occurred.

TABLE II

Effect of Phosphate on Formation of Protein

Protein formed	Protein formed
50 γ	Basal medium,* no phosphate
150	Basal medium, with phosphate
200	Basal medium**+pyruvate***, no phosphate
500	Basal medium+pyruvate, with phosphate

* Conditions, same as Table I, except that phosphate was omitted.
** Glucose 1.0 g./l.
*** Pyruvate 0.5 g./l.
Incubated 2 hours at 30°, pH 7.8.

Effects of Various Substances on Protein Formation—The gland tissues were incubated in a culture medium containing the test substances for 1 hour at 30°. These substances were dissolved in culture medium and the pH of solution was adjusted to 8.0 prior to use. The amount of protein formed was determined as described above. The results are summerized in Table III.

The protein formation was markedly stimulated by the addition of α-keto acids, such as pyruvate, phosphoenolpyruvate, oxalacetate, and α-ketoglutarate. ATP at the concentration of 0.002 to 0.003 M was as

TABLE III

Effect of Various Substances on Protein Formation in Excised Silkgland

Medium	Substance added	Concentration	Protein formed
Physiological solution		—	140 γ
Basal medium*	None	—	180
Basal medium	Pyruvic acid	0.003 M	480
,,	Lactic acid	,,	490
,,	Oxalacetic acid	,,	460
,,	Malic acid	,,	380
,,	Fumaric acid	,,	300
,,	Succinic acid	,,	400
,,	α-Ketoglutaric acid	,,	480
,,	Citric acid	,,	400
,,	Phosphoenol pyruvic acid	,,	460
,,	Phenylpyruvic acid	,,	160
,,	Glucose-1-phosphate	,,	340
,,	ATP	,,	460
,,	Vitamins B_1, B_2, B_6**		250
,,	Ascorbic acid	0.01 M	400
,,	Pantothenic acid	0.002 M	460
,,	Choline•HCl	0.003 M	200
,,	B_{12}	50 γ/lit.	350
,,	Pyruvic acid+ 2,4-dinitrophenol	0.0008 M	180
,,	Oxalacetic acid+ 2,4-dinitrophenol	0.0008 M	210
,,	Pyruvic acid+NaF	0.001 M	150

Each value is average of about twenty determination.

* The composition was given in Table I, except that 1.0 g./l. glucose was added.

** B_1•HCl 18 mg./dl.; B_2 1.5 mg./dl.; B_6 60 mg./dl.

effective as α-keto acids. Succinate, citrate, malate, and fumarate were less effective than α-keto acids.

Of the vitamines tested, pantothenic and ascorbic acids were evidently positive. Effects of thiamine, riboflavin, pyridoxine and choline hydrochloride on protein formation were not recognized.

Inhibitors: 2,4-dinitrophenol ($0.8\times10^{-3}M$) and sodium fluoride ($1\times$

$10^{-3}M$) inhibited almost completely the protein formation. The stimulating effect of α-keto acids was inhibited by these two drugs. Sodium cyanide (10^{-2}, $10^{-3}\,M$) also inhibited the protein formation, but the inhibitory effect was not consistent in twenty determinations.

Characteristics of Protein Formed—The protein formed gave a purple biuret reaction and was precipitated by mercuric chloride, trichloracetic acid, picric acid and alcohol. By paper chromatographical procedure the amino acid composition of this protein was shown to be similar to that of sericin, which was considered to be synthesized in the middle silkgland.

DISCUSSION

As described above, we have provided an evidence that the silkgland tissue could be employed as a suitable material for studing the process of protein synthesis. From the data presented, it is evident that the necessary conditions for increasing protein formation are well in agreement with the conditions which are generally considered to stimulate metabolism of tissue. On the other hand, protein formation was markedly inhibited by 2,4-dinitrophenol and sodium fluoride. These observations support the view that the increase in protein secretion represents a real synthesis of protein. Evidence that the conditions which stimulated protein formation by gland tissue were not of degradation of the tissue cells, is also shown from the experiments on the respiration of gland tissue. The oxygen uptake by silkgland in the culture medium was increased almost linearly during two hours. Addition of pyruvate to basal medium caused a slight increase of oxygen uptake (Fig. 6).

It is of interest to point out that the stimulating effect of pyruvate is considerably greater for protein formation than for the respiration of gland tissue. From the results in this paper it seems that α-keto acids are not only utilized for the synthesis of amino acids, but they have promoting effect on the reaction of protein synthesis in the gland tissue.

As shown in Table III, the average value of protein obtained for a silk gland was 140 with the physiological solution (or the presence of inhibitors). This amount of protein may be regarded as a blank value in the present work. This protein might be derived from a globular silkprotein, pre-existing in the gland tissue. Therefore, it seems more reasonable to deduct 140 from the experimental values obtained in each determination to estimate a net amount of synthesized protein.

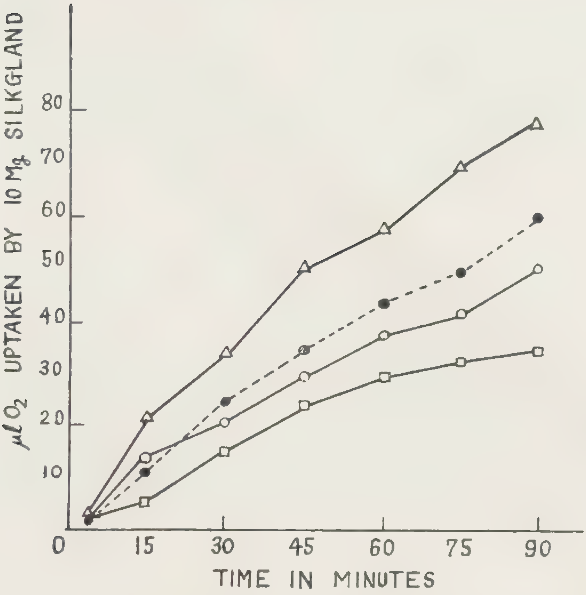


FIG. 6. Effect of pH on 0_2 uptake by silk gland

△ Basal medium, pH 7.2
○ ,, pH 7.8
□ ,, pH 8.0
● ,, + 0.003 *M* pyruvate, pH 7.8

We wish to thank Mr. K. Noguchi of the Experimental Sericultural station of Miyagi for kindly supplying the silkworm for this study and we are indebted to Miss. A. Kikuchi for her assistance in carrying out the experiment.

SUMMARY

1. A method was described for measuring the formation of protein by silkgland of silkworm *in vitro.*

2. The optimum pH for the reaction of protein formation by silkgland was found to be between 7.8 and 8.5, with a peak at pH 8.0.

3. The reaction was stimulated by the addition of inorganic phosphate.

4. The effect of various substances were studied. A marked stimulation was observed with α-keto acids (such as pyruvate, oxalacetate, α-ketoglutarate), lactate, pantothenic acid and ascorbic acid.

5. NaF and 2,4-dinitrophenol were found to inhibit the reaction.

6. The protein formed gave a purple biuret reaction and was precipitated by mercuric chloride, trichloracetic acid, picric acid, and alcohol.

7. From the above observations it is suggested that silkgland tissues synthesize protein *in vitro* under suitable conditions.

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ÜBER PFLANZENPROTEASEN

V.[1] ZUR SPEZIFITÄT DER KRYSTALLISIERTEN PROTEINASE VON BACILLUS SUBTILIS*

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(Der Schriftleitung zugegangen am Februar 8, 1955)

Die extrazellulären Proteasen der Bakterien wurden zuerst von H. Bitter, L. Brunton und Cl. Fermi (*2*) aufgefunden, und seither ihre Existenz wurde von mehreren Forschern (*3*) endgültig bewiesen. Aber in Bezug auf die Stellung dieser Exoenzyme im Proteasensystem sowie auf ihren genetischen Zusammenhang mit der Endoproteasen der Bakterien gehen bis heute noch mehrere verschiedene Ansichten voneinander (*4*). Um einen sicheren Aufschluss dafür zu geben, ist es benötigt, ein möglichst einheitlishes Enzym rein darzustellen. Zwar vor kurzem wurde eine Proteinase von S. D. Elliott (*5*) aus *Streptococcus*, von A. Güntelberg *et al.* (*6*) aus *Bacillus subtilis*, und von M. Miyake *et al.* (*7*) aus *Bacillus mesentericus* in krystallisiertem Zustande gewonnen. In letzterer Zeit ist es auch B. Hagihara (*8*) im Okunukischen Laboratorium der Universität zu Osaka gelungen, eine Proteinase aus den Kulturfiltraten von *Bac. subtilis* unter Verwendung der Chromatographie mit Kation-Austauscher und der nachfolgenden Aussalzung sich leicht auskrystallisieren zu lassen. Im Anschluss an der vorigen Mitteilung (*1*) erschien es mir sehr wichtig, die Wirkungen solches Enzyms kennenzulernen. Durch die wohlwollende Übersendung des Krystallenzyms von Prof. K. Okunuki wurde es ermöglicht, diese Aufgabe mir anzustellen.

Zur Charakterisierung der enzymatischen Natur des krystallinen Präparates habe ich zuerst die pH-Abhängigkeit der Aktivität gegen Gelatine studiert. Die Versuche ergaben, dass das Enzym die Gelatine in optimalem pH-Bereiche 7.0–8.0 anzugreifen imstande ist. Daraus geht es hervor, dass das krystallisierte Enzym als ein spezifisches, von dem bei pH 5.0–6.0 optimal wirkenden Endoenzym ganz verschiedenes Ferment darstellt. Darauf habe ich die Spaltungsversuche mit Gelatine, Casein and Clupein, in An- und Abwesenheit von Blausäure oder Cystein bei pH 9.0–8.0 durchgeführt. Hierbei ergab es sich, dass die Aktivität der Proteinase gegen die Proteine im allgemeinen von diesen Zusatzstoffen gar nicht beeinflusst werden kann. Auf Grund der von anderen Forschern (*9*) dargelegten Feststellung liegt es nahe anzunehmen, dass unsere *Subtilis*-Proteinase in vollaktiviertem Zustande vorbereitet ist.

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* Der Inhalt dieser Arbeit wurde am 27. Oktober 1954 in der Generalversammlung der Botanischen Gesellschaft von Japan, im Hörsaal der Universität zu Kyoto kurz mitgeteilt.

Es erhebt sich nun die Frage, was für Atomgruppe im Substrat für den Angriff der *Subtilis*-Proteinase spezifisch eingestellt sei. Zur Aufklärung dieser Frage habe ich die folgenden Polypeptide und Diketopiperazine in dieselben Spaltungsversuche hineinbezogen: DL-Leucyglycin, Glycyl-L-tyrosin, DL-Leucylglycylglycin, Benzoylglycin, Benzoylglycylglycin, Acetylglycylglycinäthylester, L-Tyrosinäthylester, L-Tyrosinamid, *O*, *N*-Diacetyl-L-tyrosin, *O*, *N*-Dibenzoyl-L-tyrosin, Tyrosinanhydrid, Pyrrolidoncarbonsäureamid, Pyrrolidoncarbonsäureanilid, Glycyl-L-glutaminsäureäthylesteranhydrid und Eisenin, d. h. L-Glutaminyl-L-glutaminsäureanhydrid-L-alanin. Unter diesen Substraten wurden die synthetischen Präparate im allgemeinen nach der Methode von E. Fischer (*10*) dargestellt, und das natürliche Eisenin wurde aus *Eisenia bicyclis* nach der Vorschrift von T. Ohira (*11*) gewonnen. Die Versuche ergaben, dass alle die Polypeptide, Pyrrolidencarbonsäurederivate und die neutralen Diketopiperazine auch im Gegenwart von Cystein doch durch das Enzym gar nicht aufgespalten werden. Die Sache verhielt sich bei dem Eisenin anders: dies erwies sich als ein geeignetes Substrat für die Proteinase.

Aus den obenerwähnten Tatbeständen drängt sich nun unwillkürlich die Vermutung auf, dass die Acidogruppen der Proteine für die Angreifbarkeit durch *Subtilis*-Proteinase eine wesentliche Rolle spielen können. Um weiter eine tiefere Einsicht in dieser Hinsicht zu gewinnen, habe ich die Spaltungsversuche mit den folgenden Ovalbuminderivaten ausgeführt: Ovalbumin (nativ.), Ovalbumin (denat.), Desamino-Ovalbumin, Phenylureido-Ovalbumin, Ovalbumin-Zephirol, Ovalbumin-Methylester, Acetyl-Ovalbumin-Zephirol und Acetyl-Ovalbumin-Metylester. Die Darstellungsverfahren und analytischen Daten dieser Präparate wurden schon früher von mir (*12*) veröffentlicht. Zur Veranschaulichung der relativen Spaltungsgrade wurde die COOH-Zunahme durch Titrationsmethode an jeder Probe gemessen, und der Wert in Prozenten des beim Ovalbumin (denat.) ergebenen umgerechnet. Die Versuche ergaben, dass die acylierten Proteine durch das Enzym mit dem Spaltugsgrad 73–85 gut aufgespalten werden, während sich die an Acidogruppen maskierten Proteine mit dem Spaltungsgrad 3.0–18.0 als schwer angreifbar erweisen. Das native Ovalbumin verhielt sich gegen das *Subtilis*-Enzym, dem Ovalbumin-Methylester ähnlich, sehr träge.

Im Zusammenhange mit diesen Tatsachen sei es hierbei hervorgehoben, dass die krystallisierte Bakterienproteinase ein Substanz von Basoprotein-Natur ist. Nach S. S. Elliott *et al.* (*13*) liegt der isoelektrische Punkt des krystallisierten *Streptococcus*-Enzyms bei pH 8.42. Im Rücksicht auf der Angabe von B. Hagihara, dass die *Subtilis*-Proteinase erst beim pH 11.3 aus saurem Adsorbens eluiert werden kann, geht es klar hervor, dass dies Enzym ein basisches Protein darstellt. Hierbei kann man wohl mit grosser Wahrscheinlichkeit annehmen, dass die sauren Atomgruppen des Substratproteins an den basischen Atomgruppen des Enzymproteins verankert werden können, um damit die Peptidbindungen des ersteren spezifisch der Spaltwirkungen des letzteren anheimzufallen. Wenn es tatsächlich der Fall sein mag, so kann man erwarten, dass die enzymatische Angreifbarkeit eines Proteins durch die vorherige Blockierung der ionisierten Atomgruppen mit anderen hockmolekularen Elektrolyten wie z. B. Farbstoffen verzögert werden könne. In Anbetracht dessen habe ich verschiedene Farbstoff-Gelatineverbindungen dargestellt und ihre Spaltbarkeit durch *Subtilis*-Proteinase untersucht. Die hierzu angewandten Substrate sind die folgenden: Gelatine, Ferrocyanwasserstoffsäure-Gelatine, $Cu(OH)_2$-Gelatine, Krystallviolett-Gelatine, Fuchsin-Gelatine, Safranin-Gelatine, Methylenblau-Gelatine, Thioninblau-Gelatine, Congorot-Gelatine und Eosin-Gelatine. Die Versuche ergaben, dass die Gelatine durch die Kuppelung mit diesen Farbstoffen ausnahmslos gegen Enzym mehr oder minder refraktär gemacht werden kann.

Bezüglich der schwereren Angreifbarkeit des nativen Ovalbumins muss man hierbei natürlich auf dem Umstand berücksichtigen, dass die —NH—CO—Bindungen im Protein, welche für den Angriff der Proteinasen spezifisch eingestellt sind, nicht stets im enzymaffinen Zustande angelagert sind. Diese Tatsache wurde von mir (*14*) bei mehreren Proteinen vollends bewiesen. Hiermit habe ich nun den Einfluss der Hitze-Denaturierung der Proteine auf ihre Spaltbarkeit durch die *Subtilis*-Proteinase geprüft. Die in diesem Versuche herangezogenen Proteine sind Rind-Hämoglobin, Ovalbumin, *Glycine*-Legumelin und Glycinin. Dabei ergab es sich, dass die wasserlöslichen Proteine wie Hämoglobin, Ovalbumin und Legumelin in nativem Zustande schwer angreifbar sind, während sie nach Denaturierung ins leicht Spaltbare verändert werden. Aber das wasserunlösliche Glycinin erwies sich sowohl in nativem als auch in denaturiertem Zustande als ein gutes Substrat für das Enzym.

EXPERIMENTELLES

Das in der vorliegenden Arbeit angewandte Enzympräparat ist das, welches von B. Hagihara aus Kulturfiltraten von *Bac. subtilis* in krystallisiertem Zustande dargestellt wurde, und in verdünntem Aceton verteilt war. Diese originale Enzymsuspension wurde mit destilliertem Wasser 10-fach verdünnt, und diese Lösung wurde als Enzymlösung angewandt.

I. Einfluss der Wasserstoffionenkonzentration und Blausäure- bzw. Cystein-Zusatzes auf die proteolytische Aktivität der Subtilis-Proteinase

(*1*) *pH-Abhängigkeit der Gelatinespaltung*—

Versuchsanstellung: In 1 ccm. Ansatz sind 60 mg. Gelatine, 0.2 ccm. Enzymlösung nebst variierender Menge der verschiedenen Säure oder des Ammoniaks als Puffer enthalten. Toluol als Antiseptikum. Versuchstemp. 30°. Einwirkungsdauer 24 Stdn. Bestimmungsmethode: Die COOH-Zunahme wurde durch Titrationsmethode mit 0.025 *N* KOH in Alkohol gemessen.

(2) *Einfluss des HCN- oder Cystein-Zusatues auf die Proteolytische Aktivität*—

Versuchsanstellung: 1 ccm. Ansatz enthielt 40 mg. Substratprotein, 0.2 ccm. Enzymlösung, variierende Menge der Blausäure oder des L-Cysteins nebst dem Ammoniakpuffer. Toluol als Antiseptikum. Temp. 30°. Versuchsdauer 24 Studen. Bestimmungsmethode: Wie oben.

Die Ergebnisse sind in folgender Tabelle angegeben.

TABELLE I

Substrat	Zusatz pro 1 ccm. Ansatz		pH	COOH-Zunahme in Äquiv. 0.025 *N* KOH
pH-Abhängigkeit				
		mM		*ccm.*
Gelatine (60 mg.)	HCl	0.04	2.0	0.00
〃 〃	CH_3COOH	0.02	4.3	1.00
〃 〃	—	—	5.6	1.60
〃 〃	NH_4OH	0.01	6.0	1.80
〃 〃	〃	0.04	7.4	2.20
〃 〃	〃	0.08	8.0	2.00
〃 ···	〃	0.10	8.6	1.80
〃 〃	〃	0.40	9.6	1.20

HCN- oder Cystein-Einfluss				
Gelatine (40 mg.)	NH_4OH	0.03	7.2	2.20
〃 〃	KCN CH_3COOH	0.02 0.01	7.2	2.00
〃 〃	L-Cystein NH_4OH	0.02 0.03	7.2	2.00
Casein 〃	NH_4OH	0.03	7.2	3.00
〃 〃	L-Cystein NH_4OH	0.02 0.03	7.2	2.90
Clupeinsulfat (40 mg.)	NH_4OH	0.05	7.2	3.00
〃 〃	L-Cystein NH_4OH	0.02 0.05	7.2	2.80

II. *Spaltbarkeit der Polypeptide und Diketopiperazine durch Subtilis-Proteinase*

Versuchsanstellung: 1 ccm. Ansatz enthielt 0.05 mM Substrat, 0.2 ccm. Enzymlösung nebst der verschiedenen Menge des Ammoniaks als Puffer, und Toluol als Antiseptikum. Temp. 30°. Versuchsdauer 24 Stdn.

Bestimmungsmenthode: Die Carboxylzunahme wurde in üblicher Weise titrimetrisch bestimmt, und der Spaltungsgrad wurde in Prozenten des 1-Peptidbindung pro Mol der Substrate gekennzeichnet. Die Ergebnisse sind in Tab. II zusammengestellt.

TABELLE II

Substrat (0.05 mM)	Zusatz als Puffer oder Aktivator		pH	COOH-Zunahme in Äquiv. 0.025 N KOH	Spaltungsgrad
		mM		*ccm.*	%
DL-Leucylglycin	NH_4OH	0.03	7.2	0.00	0.0
〃	〃 L-Cystein	0.06 0.02	7.4	0.00	0.0
Glycyl-L-tyrosin	NH_4OH	0.03	8.0	0.00	0.0
DL-Leucylglycylglycin	〃	0.03	7.0	0.00	0.0
〃	〃 L-Cystein	0.03 0.02	7.2	0.00	0.0
Benzoylglycin	NH_4OH	0.06	5.0	0.00	0.0
Benzoylglycylglycin	〃	0.06	5.0	0.00	0.0
〃	〃 L-Cystein	0.12 0.02	7.0	0.00	0.0

Substrat (0.05 mM)	Zusatz als Puffer oder Aktivator		pH	COOH-Zunahme in Äquiv. 0.025 *N* KOH	Spaltungsgrad
		mM		*ccm.*	%
Acetylglycylglycinäthylester	NH_4OH	0.02	7.2	0.00	0.0
L-Tyrosinäthylester-HCl	〃	0.05	8.0	0.00	0.0
L-Tyrosinamid-HCl	〃	0.05	7.8	0.00	0.0
O,N-Diacetyl-L-tyrosin	〃	0.05	6.8	0.00	0.0
O,N-Dibenzoyl-L-tyrosin	〃	0.05	7.2	0.00	0.0
Tyrosinanhydrid	〃	0.025	8.0	0.00	0.0
Pyrrolidoncarbonsäureamid	〃	0.025	8.4	0.00	0.0
Pyrrolidoncarbonsäureanilid	〃	0.025	8.6	0.00	0.0
Glycyl-L-glutaminsäureäthylesteranhydrid	〃	0.025	8.2	0.00	0.0
Eisenin	〃	0.08	6.8	0.70	35.0
〃	〃	0.10	7.6	1.40	70.0
〃	〃	0.16	8.4	1.20	60.0
〃	〃	0.20	9.2	0.80	40.0

III. Bestimmung der enzymaffinen Atomgruppen im Substratprotein

(1) Spaltbarkeit der substituierten Ovalbuminderivate—

Substratproteine: Die hierzu angewandten Substrate sind die Ovalbuminderivate’ deren präparative Verfahren sowie ihre analytischen Daten in früherer Mitteilung (*12*) beschreieben wurden.

Versuchsanstellung: In 1 ccm. Ansatz sind 40 mg. Protein, 0.2 ccm. Enzymlösung, 0.05 mM NH_4OH (pH 7.0–8.0) als Puffer und Toluol als Antiseptikum enthalten. Temp. 30°. Einwirkungsdauer 24 Stunden.

Bestimmungsmethode: Die Carboxylzunahme wurde durch Titrationsmethode bestimmt. Der Spaltungsgrad wurde in Prozenten des Peptid-N angegeben. Zur Ermittelung der relativen Spaltungsgrade wurde derselbe beim denaturierten Ovalbumin als 100 umgerechnet.

(2) Spaltbarkeit der Farbstoff-Gelatineverbindungen—

Darstellung der Substrate: 2–3 g. Gelatine wurden in 10-fach Menge des Wassers gelöst, und darauf wurde die Farbstofflösung (0.2–0.3 g. Farbstoff in Wasser) versetzt. Daraus wurde das gefärbte Protein mit Alkohol gefällt. Der Fällungskörper wurde mit warmem Alkohol digeriert, auf Glasfilter gesammelt, mit Alkohol und Äther nachgewaschen, und im Exsikkator über Schwefelsäure getrocknet. Das Ferrocyanwasserstoffsäure-Gelatinekomplex wurde beim Zusatz dieser Säure auf Gelatine-lösung als eine unlösliche Substanz gewonnen. Zur Darstellung der $Cu(OH)_2$-Gelatineverbindung wurde die Gelatine mit $Cu(OH)_2$ in alkalischer Reaktion versetzt, und daraus wurde das Gelatinekomplex mit Alkohol gefällt. Der Peptid-N der Präparate wurde durch die NH_2-Zunahme nach vollständiger Hydrolyse mit 20-proz. Schwefelsäure ermittelt.

Versuchsanstellung: 1 ccm. Ansatz enthielt 60 mg. Protein, 0.2 ccm. Enzymlösung, 0.02 mM NH_4OH (pH 6.8-7.4) als Puffer und Toluol als Antiseptikum. Temp. 30°. Versuchsdauer 24 Stunden.

Bestimmung der Spaltungsgrade: Die NH_2-Zunahme wurde nach van Slyke bestimmt, und der Wert wurde in Prozenten vom Peptid-N des Substratproteins als Spaltungsgrad ausgedrückt. Der relative Spaltungsgrad wurde in Prozenten von dem bei der ungefärbten Gelatine ergebenen Wert berechnet.

(3) *Einfluss der Hitze-Denaturierung der Proteine auf ihre enzymatische Spaltbarkeit*—

Versuchsanstellung: In 1 ccm. Ansatz sind 100 mg. Protein und 0.2 ccm. Enzymlösung nebst 0.04 mM NH_4OH (pH 7.0–7.6) enthalten. Zur Denaturierung wurde das Substratprotein vorher mit wenig Wasser 20 Minuten auf Wasserbade gekocht. Toluol als Antiseptikum. Temp. 30°. Einwirkungsdauer 24 Stunden.

Bestimmungsmethode: Die Carboxylzunahme wurde durch Titrationsmethode bestimmt. Zur Veranschaulichung der Denaturierungseinflüsse wurde der Spaltungsgrad des nativen Proteins in Prozenten des beim denaturierten Protein ergebenen Wertes als Spaltungsquotient des nativen Proteins angegeben.

Die Ergebnisse sind in Tabelle III zusammengestellt.

TABELLE III

Substratprotein			COOH-Zunahme in Äquiv. 0.025 *N* KOH	Spaltungsgrad (% vom Peptid-N)	Relativ. Spaltungsgrad
Ovalbuminderivat (40 mg.)	Gesamt-N	Peptid-N			
	%	%	ccm.	%	%
Ovalbumin (nativ.)	15.89	11.87	0.75	5.5	20.8
Ovalbumin (denat.)	〃	〃	3.60	26.5	100.0
Desamino-Ovalbumin	15.20	11.37	2.80	22.4	84.5
Phenylureido-Ovalbumin	14.16	10.29	2.25	19.2	72.5
Ovalbumin-Zephirol	14.12	10.54	0.10	0.8	3.0
Ovalbumin-Methylester	14.69	11.52	0.60	4.5	17.0
Acetyl-Ovalbumin-Zephirol	12.25	10.83	0.50	4.0	15.3
Acetyl-Ovalbumin-Methylester	13.16	10.83	0.60	4.8	18.1

Substratprotein			NH$_2$-N-Zunahme	Spaltudgs-grad (% vom Peptid-N)	Relativ. Spaltungs-grad
Gelatinederivat (60 mg.)	Gesamt-N	Peptid-N			
	%	%	mg.	%	%
Gelatine	16.46	11.20	1.1500	17.1	100.0
$H_4[Fe(CN)_6]$-Gelatine	—	8.80	0.6904	13.1	76.6
$Cu(OH)_2$-Gelatine	—	10.67	0.6330	10.3	60.2
Krystallviolett-Gelatine	—	11.00	0.8628	13.1	76.6
Fuchshin-Gelatine	—	11.05	0.9202	13.9	81.2
Safranin-Gelatine	—	11.48	1.0351	15.0	87.7
Methylenblau-Gelatine	—	11.48	1.0351	15.0	87.7
Thioninblau-Gelatine	—	11.71	0.9202	13.1	76.6
Congorot-Gelatine	—	11.05	0.9777	14.7	85.9
Eosin-Gelatine	—	11.05	0.8628	13.0	76.0

Einfluss der Hitze-Denaturierung

Substratprotein (100 mg.)	COOH-Zunahme in Äquiv. 0.025 N KOH		Spaltungs-quotient des nativen Proteins
	Ungekochtes	Gekochtes	
	ccm.	ccm.	%
Rind-Hämoglobin	0.25	5.55	4.5
Ovalbumin	0.90	3.65	25.0
Glycine-Legumelin	0.00	3.80	0.0
Glycinin	6.20	6.10	101.0

ZUSAMMENFASSUNG

An Hand der krystallisierten *Subtilis*-Proteinase habe ich die Spaltungsversuche mit den folgenden Substraten ausgeführt: Gelatine, Ovalbumin, Hämoglobin, Clupein, Glycinin, Legumelin, mehrere Polypeptide, Diketopiperazine und Pyrrolidoncarbonsäurederivate. Zur Ermittelung der Spaltungsgrade wurde die COOH-Zunahme durch Titrationsmethode gemessen. Die Versuche ergaben, dass sich das Enzym als eine reine Proteinase erweist, also es völlig frei von Dipeptidase, Aminopolypeptidase und Carboxypolypeptidase ist. Es wurde festgestellt, dass sowohl das Protein als auch das Eisenin durch das Enzym in optimalem pH-Bereiche 7.0–8.0, auch in Abwesenheit von Blausäure oder Cystein gut angegriffen werden kann. Um irgendwelche enzymaffinen Atomgruppen im Substratprotein zu entdecken, habe ich die verschiedenen Ovalbuminderivate und die mehreren Farbstoff-Gelatineverbindungen zum vergleichenden Spaltungs versuche unterworfen, mit dem Ergebnis, dass die Bakterienproteinase spezifisch für die Acidogruppen des Proteinmoleküls eingestellt ist. Daraus hat es sich herausgestellt, dass das krystalline *Subtilis*-Enzym der Kategorie der Proteinasen von Papain- oder Trypsin-Typus angehört.

Zum Schluss möchte ich meinem verehrten Kollege, Herrn Dr. K. Okunuki, für seine liebenswürdige Unterstützung mit dem Krystallenzym meinen herzlichen Dank aussprechen. Dem Unterrichtsministerium bin ich dankbar für die Gewährung des Forschungsstipendiums.

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METABOLISM OF HISTIDINE. VI

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It was previously reported by us (*1*) that L-histidine deaminase purified from bacteria is a metalprotein which requires mercury, cadmium or zinc ion. Prior to this, Kumagai (*2*) observed with guinea pig liver enzyme that reduced glutathione (GSH) activates the decomposition of L-histidine, and considered that GSH is a co-enzyme of L-histidine deaminase.

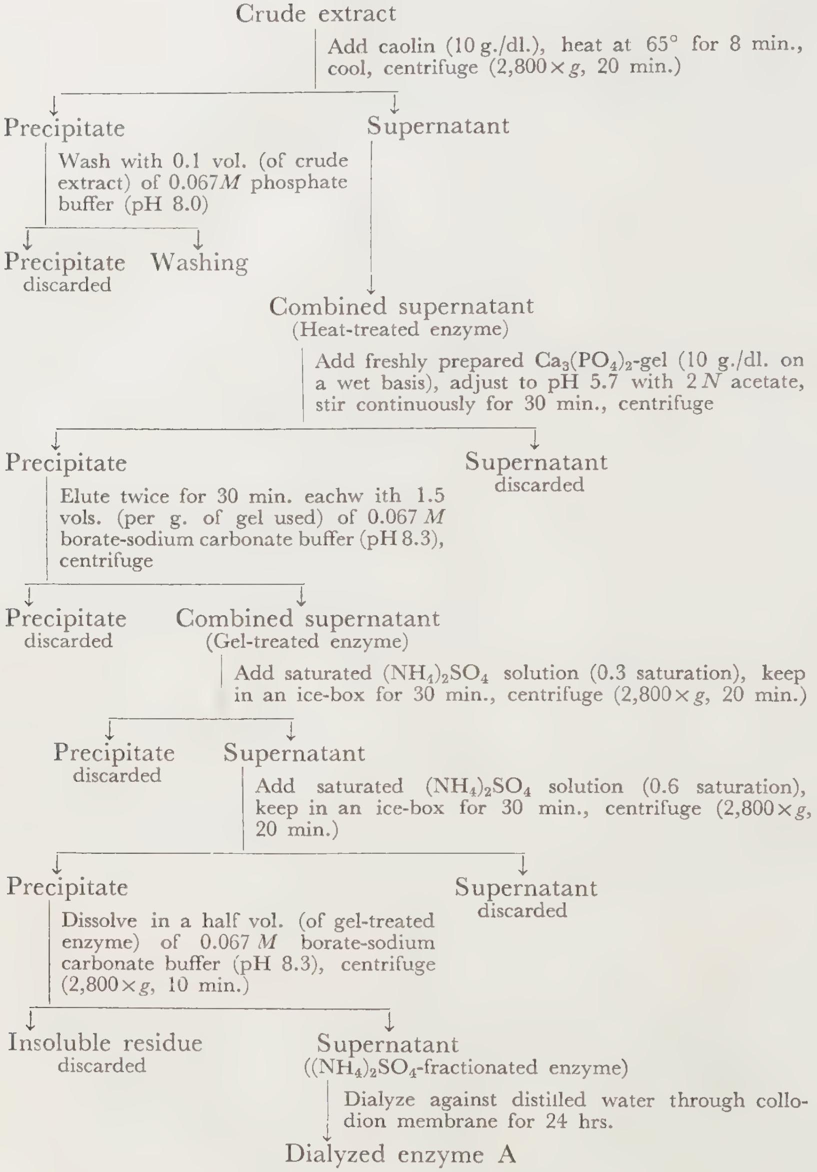
Later, Ichihara *et al.* (*3*) reported that the lost activity of L-histidine deaminase from rabbit liver is not entirely restored by the sole addition of GSH but completely by folic acid together with GSH. More later, supplementing cobalt ion, Ichihara *et al.* (*4*) asserted that folic acid, cobalt ion and GSH constitute the co-enzyme moiety of L-histidine deaminase.

In this paper evidences are presented that folic acid has no effect on the enzyme action of L-histidine deaminase purified from guinea pig liver, and that GSH is not a specific co-factor of the enzyme but a nonspecific SH-activator, that is, the other SH-compounds such as thioglycollate and cysteine are found as effective as GSH, and further, that cadmium ion together with the one of the above SH-compounds participates in the enzymatic deamination of L-histidine.

MATERIALS AND METHODS

Preparation of the Enzyme Extract—Guinea pigs weighing about 400 g. were used as experimental animals, which were injected intramuscularly 1 mg. of L-histidine monohydrochloride emulsified in rape oil per g. of body weight, and sacrificed about twenty hours later, then the fresh livers were homogenized with three volumes (v./w.) of ice-cold 0.067 M phosphate buffer (pH 8.0). The homogenate was centrifuged at $2{,}800 \times g$ for 30 minutes, and the supernatant was subjected as the "crude extract" to the further treatments of purification.

SCHEME I



Crude extract

Add caolin (10 g./dl.), heat at 65° for 8 min., cool, centrifuge (2,800 × *g*, 20 min.)

Precipitate

Wash with 0.1 vol. (of crude extract) of 0.067 *M* phosphate buffer (pH 8.0)

Precipitate discarded

Washing

Supernatant

Combined supernatant (Heat-treated enzyme)

Add freshly prepared $Ca_3(PO_4)_2$-gel (10 g./dl. on a wet basis), adjust to pH 5.7 with 2 *N* acetate, stir continuously for 30 min., centrifuge

Precipitate

Elute twice for 30 min. eachw ith 1.5 vols. (per g. of gel used) of 0.067 *M* borate-sodium carbonate buffer (pH 8.3), centrifuge

Supernatant discarded

Precipitate discarded

Combined supernatant (Gel-treated enzyme)

Add saturated $(NH_4)_2SO_4$ solution (0.3 saturation), keep in an ice-box for 30 min., centrifuge (2,800 × *g*, 20 min.)

Precipitate discarded

Supernatant

Add saturated $(NH_4)_2SO_4$ solution (0.6 saturation), keep in an ice-box for 30 min., centrifuge (2,800 × *g*, 20 min.)

Precipitate

Dissolve in a half vol. (of gel-treated enzyme) of 0.067 *M* borate-sodium carbonate buffer (pH 8.3), centrifuge (2,800 × *g*, 10 min.)

Supernatant discarded

Insoluble residue discarded

Supernatant ($(NH_4)_2SO_4$-fractionated enzyme)

Dialyze against distilled water through collodion membrane for 24 hrs.

Dialyzed enzyme A

Experimental Apparatus—Sealed but not evacuated Thunberg tube was used to determine ammonia liberated together with L-histidine decomposed and urocanic acid formed. After incubation, trichloracetic acid contained in a side chamber of the Thunberg tube was combined with the reaction mixture in a main chamber, and shaken vigorously to fix ammonia. Then, aliquots of centrifuged supernatant were used to determine L-histidine, ammonia and urocanic acid.

Estimation of L-Histidine—L-Histidine was determined by Sera's bromine reaction (*5*), the purple coloration was measured colorimetrically at 540 mμ with Coleman Model 6A spectrophotometer.

Estimation of Ammonia—Ammonia which was liberated by deamination of L-histidine was estimated by Conway's microdiffusion method.

Estimation of Urocanic Aaid—Urocanic acid was determined spectrophotometrically at 265 mμ with Beckman spectrophotometer Model DU.

Estimation of Protein—Protein was estimated by Folin-Ciocalteu's phenol reaction (*6*); the blue coloration was measured colorimetrically at 700 mμ with Coleman Model 6A spectrophotometer.

RESULTS

Purification of the Enzyme (*Process A*)—L-Histidine deaminase was purified as shown in SCHEME 1. The purified "dialyzed enzyme A" thus

TABLE I

	Volume	Total protein	Specific* activity	Yield
	ml.	*mg.*		%
Crude enzyme	180	1758.6	0.51	100.0
Heat-treated enzyme	165	224.1	1.30	47.7
Gel-treated enzyme	65	35.2	6.00	23.6
$(NH_4)_2SO_4$-fractionated enzyme	30	13.8	8.22	12.7
Dialyzed enzyme	42	11.6	8.25	9.3

Experimental conditions: enzyme solution at each step of purification, 1.0 ml.; 5×10^{-2} *M* L-histidine, 0.2 ml.; 0.2 *M* TRIS buffer (pH 8.9), 0.7 ml.; final volume, 2.0 ml. with 0.1 ml. distilled water. After incubation at 37° for 1 hour, 0.2 ml. of 50 per cent trichloracetic acid was added to stop the reaction. Aliquots of the centrifuged supernatants were subjected to the estimation of L-histidine.

* Specific activity represents micromoles of L-histidine decomposed by 1 mg. of protein at 37° for 1 hour.

Table II

Inaibitor	Final concentration	L-Histidine decomposed	Inhibition ratio	Ammonia liberated	Inhibition ratio	Urocanic acid formed	Inhibition ratio
	M	μ*M*	%	μ*M*	%	μ*M*	%
EDTA	—	5.90	94.9	5.72	100.0	5.64	100.0
	10^{-3}	0.30		0.00		0.00	
8-Hydroxyquinoline	—	Incapable of colorimetric determination		5.72	78.2	5.64	78.4
	2×10^{-3}			1.16		1.22	
o-Phenanthroline	—	Incapable of colorimetric determination		5.72	71.2	5.64	69.0
	2×10^{-3}			1.60		1.75	
α, α′-Dipyridyl	—	5.90	39.8	5.72	38.4	5.64	36.0
	2×10^{-3}	3.54		3.54		3.61	
KCN	—	5.90	6.8	5.72	9.5	5.64	7.5
	10^{-2}	5.51		5.15		5.22	
Hydroxylamine	—	5.90	78.0	5.72	86.1	5.64	81.0
	5×10^{-3}	1.34		0.80		1.08	
Semicarbazide	—	2.89	0.7	2.84	1.6	2.96	2.0
	5×10^{-3}	2.87		2.80		2.90	
p-Chloromercuribenzoate	—	10.60	100.0	9.81	100.0	9.63	100.0
	10^{-3}	0.00		0.00		0.00	
Monoiodacetate	—	8.74	29.0	8.65	28.0	8.62	29.2
	5×10^{-3}	6.67		6.25		6.10	

Experimental conditions: Dialyzed enzyme A (pH adjusted to 8.7 with 0.1 *N* NaOH), 2.0 ml.; 5×10^{-2} *M* L-histidine, 0.4 ml.; 0.2 *M* TRIS buffer (pH 8.7), 1.0 ml.; inhibitors, in respective concentration; final volume, 4.0 ml. with adequate amounts of distilled water.

After incubation at 37° for 2 hours in the main chamber of Thunberg tube, 0.6 ml. of 40 per cent trichloracetid acid was tipped in from the side chamber to stop the reaction and to fix ammonia.

obtained was a faintly yellowish clear solution and had no urocanic acid decomposing activity which had been removed at the step of heat-treatment. Its specific activity was about twenty folds as high as that of the crude extract, and no decrease in enzymatic activity could be observed through further dialysis. Protein content, specific activity and yield at each step of purification are summarized in Table I.

Effects of Inhibitors—Each of $10^{-3}\,M$ ethylenediaminetetraacetate (EDTA), $2\times10^{-3}\,M$ 8-hydroxyquinoline, $2\times10^{-3}\,M$ *o*-phenanthroline, $10^{-2}\,M$ KCN, $5\times10^{-3}\,M$ hydroxylamine, $10^{-3}\,M$ *p*-chloromercuribenzoate and $5\times10^{-3}\,M$ monoiodacetate completely or significantly inhibited the enzyme activity as shown in Table II.

These results suggest that L-histidine deaminase may require some metallic ion and free SH-group for its activity.

Effects of Metallic Ions—Effects of various metallic ions were examined with the "dialyzed enzyme A". As shown in Table III, Ag^+, Hg^{++}, Ni^{++}, Pb^{++}, Fe^{+++}, Cu^{++}, Sn^{++}, Ba^{++} and Sr^{++} were inhibitory in varying degrees and Al^{+++}, Ca^{++}, Mg^{++}, Co^{++}, Cr^{++}, Fe^{++}, Zn^{++} and Li^+ were without effect on the enzyme action, whereas Cd^{++} exhibited a marked stimulatory effect. Concentration optimum of Cd^{++} was found $3.3\times10^{-4}\,M$ under the experimetal conditions employed as shown in Fig. 1.

TABLE III

Metallic ion ($3.3\times10^{-4}\,M$)	L-Histidine decomposed	Ammonia liberated	Urocanic acid formed	Inhibition ratio
	μM	μM	μM	%
—	4.07	3.96	4.01	0.0
Ag^+	0.00	0.00	0.00	100.0
Hg^{++}	0.00	0.00	0.00	100.0
Ni^{++}	2.12	2.14	2.10	47.1
Pb^{++}	2.76	2.68	2.60	33.3
Fe^{+++}	2.98	2.81	2.88	28.1
Cu^{++}	3.22	3.17	3.12	20.2
Sn^{++}	3.38	3.30	3.32	17.2
Ba^{++}	3.61	3.61	3.64	9.8
Sr^{++}	3.71	3.62	3.65	9.0

Experimental conditions were the same as described in Table II with the exception that inhibitors were substituted for metallic ions.

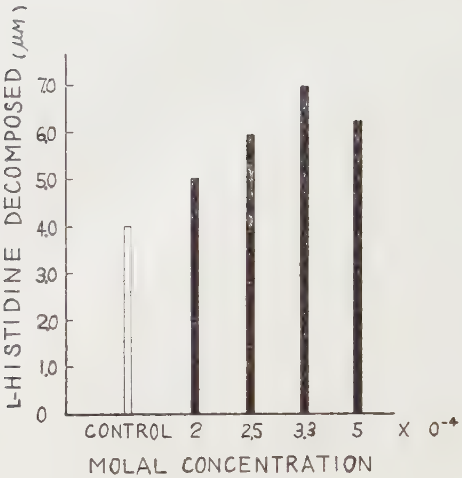


Fig. 1. Experimental conditions: Dialyzed enzyme A (pH adjusted to 8.7 with 0.1N NaOH), 1.0 ml.; 5×10^{-2} M L-histidine, 0.2 ml.; 0.2M TRIS buffer, 0.5 ml.; $CdSO_4$ in varying concentrations; final volume, 2.0 ml. with adequate amounts of distilled water.

After incubation at 37° for 2 hours, 0.2 ml. of 50 per cent trichloracetic acid was added to stop the reaction. Aliquots of the centrifuged supernatants were subjected to the estimation of L-histidine.

TABLE IV

Additions	L-Histidine decomposed	Activation ratio
	μM	
None	4.6	1.00
GSH	4.9	1.07
Cd^{++}	5.8	1.27
Cd^{++} and GSH	7.8	1.68
Cd^{++} and thioglycollate	7.8	1.68
Cd^{++} and cysteine	7.4	1.60

Experimental conditions: dialyzed enzyme A (pH adjusted to 8.7 with 0.1 N NaOH), 2.0 ml.; 5×10^{-2} M L-histidine, 0.4 ml.; 0.2 M TRIS buffer (pH 8.7), 1.0 ml.; 6.6×10^{-3} M $CdSO_4$, 0.2 ml.; 4×10^{-2} M GSH, thioglycollate or cysteine, 0.2 ml.; final volume, 4.0 ml. with adequate amounts of distilled water. After incubation at 37° for 2 hours, 0.6 ml. of 40 per cent trichloracetic acid was added to stop the reaction. Aliquots of centrifuged supernatants were subjected to the estimation of L-histidine.

Effects of SH-compounds—When "dialyzed enzyme A" was supplemented with Cd^{++} together with GSH, thioglycollate or cysteine, much more enhancement of the enzyme activity was observed than supplemented separately as shown in Table IV.

Effects of the Othes Substances—Vitamin C and folic acid were found to have no effects as shown in Tables V and VI. Neither boiled juice of a fresh liver homogenate nor HCl-extract of liver ash were effective on the enzyme activity.

Table V

Additions	L-Histidine decomposed	Activation ratio
	μM	
None	8.4	1.00
Cd^{++}	10.3	1.23
Vitamin C	8.5	1.01
Cd^{++} and vitamin C	10.3	1.23
Cd^{++} and GSH	14.3	1.70
Cd^{++}, GSH and vitamin C	14.4	1.71

Experimental conditions were the same as described in Table IV with the exception of additional supplementation of 10^{-1} M vitamin C, 0.2 ml.

Table VI

Additions	L-Histidine decomposed	Activation ratio
	μM	
None	5.0	1.00
Cd^{++}	9.2	1.84
Folic acid	5.0	1.00
Cd^{++} and folic acid	9.2	1.84
Cd^{++} and GSH	13.6	2.76
GSH and folic acid	5.3	1.06
Cd^{++}, GSH and folic acid	13.0	2.66

Experimental conditions were the same as described in Table IV with the exception of additional supplementation of 1 per cent solution of folic acid, 0.2 ml.

Another Purification of the Enzyme (*Process B*)—As mentioned above, "dialyzed enzyme A" obtained by the purification "process A" maintained its activity even after a long period of dialysis. In studying purification further, a preparation which lost its activity on dialysis was obtained by an isoelectric precipitation following heat treatment. That is, pH of the heat-treated enzyme was adjusted to 5.0 with $2N$ acetate, and the precipitates formed were separated by centrifugation at $2{,}800 \times g$ for 15 minutes. The supernatant was discarded and the precipitate was dissolved in $0.067M$ phosphate buffer (pH 8.0). This "isoelectrically precipitated enzyme" was subjected successively to the following purification procedures: tricalcium phosphate gel treatment, ammonium sulfate fractionation and dialysis against distilled water. The enzyme fraction thus obtained, "dialyze enzyme B′", was inactivated through dialysis and reactivated by Cd^{++} together with GSH, but, as indicated in Table VII (1), its yield was so poor that, consulting the effects of SH-compounds described above, another purification was attempted by adding thioglycollate at each step of purification.

The entire purification process "Process B", is illustrated in SCHEME II. Protein content, specific activity and yield are summarized in Table VII (2.)

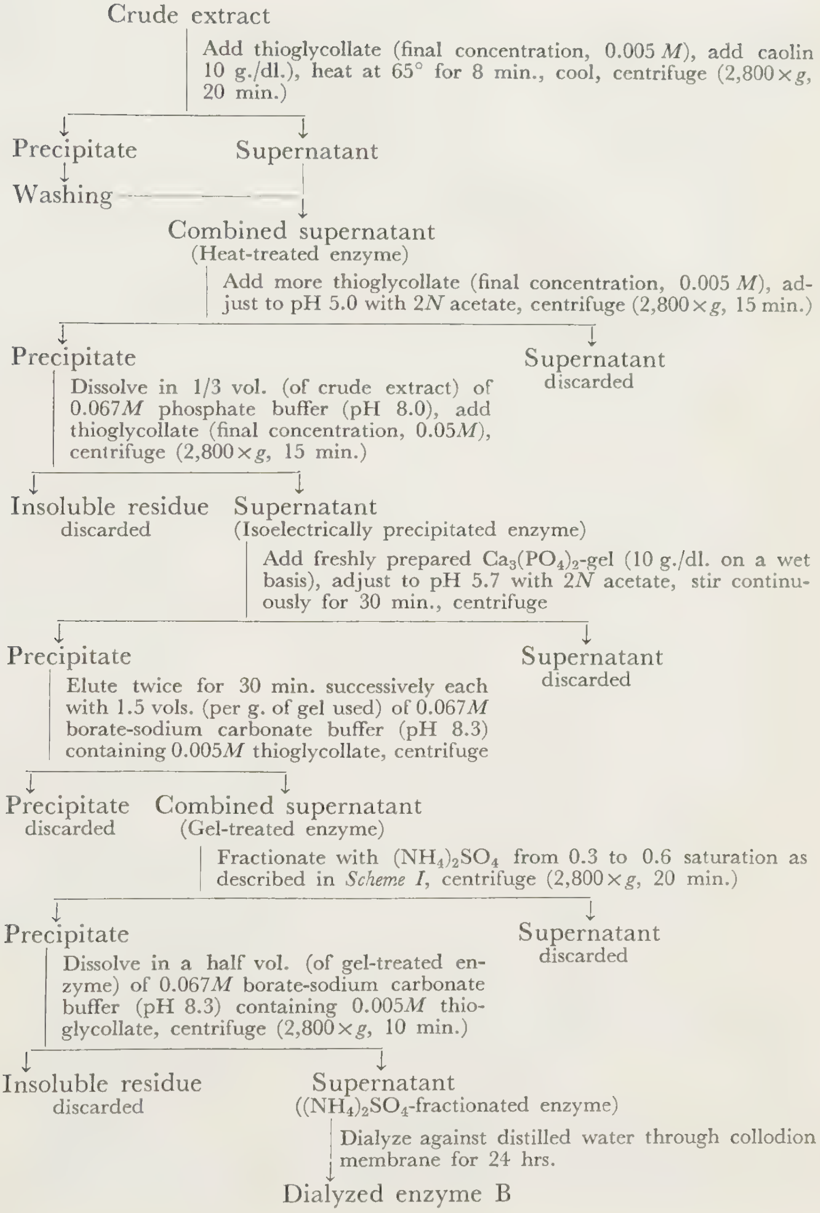
TABLE VII (1)

Enzyme	Volume	Total protein	Specific activity	Yield
	ml.	*mg.*		%
Crude extract	150	2360.1	0.39	100.0
Heat-treated enzyme	132	352.2	1.37	52.5
Isoelectrically precipitated enzyme	70	52.0	3.68	21.0
Gel-treated enzyme	30	18.1	5.75	11.3
$(NH_4)_2SO_4$-fractionated enzyme	15	9.5	2.08	
			(Cd^{++}, GSH) 8.71	9.0
Dialyzed enzyme B'	21	5.9	0.00	
			(Cd^{++}, GSH) 11.92	7.6

Participatiom of Cd^{++} and SH-compounds in the Enzymatic Reaction—"Dialyzed enzyme B", which had been completely inactivated through 24 hours dialysis, regained its activity most intensely by the addition of Cd^{++} together with a SH-compound such as GSH, thioglycollate or cysteine as shown in Table VIII.

pH Optimum and Michaelis Constant—Employing the "dialysed enzyme B", pH optimum of L-histidine deaminase was determined to be 8.7 as shown in Fig. 2.

SCHEME II



Crude extract

Add thioglycollate (final concentration, 0.005 *M*), add caolin 10 g./dl.), heat at 65° for 8 min., cool, centrifuge (2,800 × *g*, 20 min.)

Precipitate → Washing

Supernatant

Combined supernatant
(Heat-treated enzyme)

Add more thioglycollate (final concentration, 0.005 *M*), adjust to pH 5.0 with 2*N* acetate, centrifuge (2,800 × *g*, 15 min.)

Precipitate

Dissolve in 1/3 vol. (of crude extract) of 0.067*M* phosphate buffer (pH 8.0), add thioglycollate (final concentration, 0.05*M*), centrifuge (2,800 × *g*, 15 min.)

Supernatant
discarded

Insoluble residue
discarded

Supernatant
(Isoelectrically precipitated enzyme)

Add freshly prepared $Ca_3(PO_4)_2$-gel (10 g./dl. on a wet basis), adjust to pH 5.7 with 2*N* acetate, stir continuously for 30 min., centrifuge

Precipitate

Elute twice for 30 min. successively each with 1.5 vols. (per g. of gel used) of 0.067*M* borate-sodium carbonate buffer (pH 8.3) containing 0.005*M* thioglycollate, centrifuge

Supernatant
discarded

Precipitate
discarded

Combined supernatant
(Gel-treated enzyme)

Fractionate with $(NH_4)_2SO_4$ from 0.3 to 0.6 saturation as described in *Scheme I*, centrifuge (2,800 × *g*, 20 min.)

Precipitate

Dissolve in a half vol. (of gel-treated enzyme) of 0.067*M* borate-sodium carbonate buffer (pH 8.3) containing 0.005*M* thioglycollate, centrifuge (2,800 × *g*, 10 min.)

Supernatant
discarded

Insoluble residue
discarded

Supernatant
($(NH_4)_2SO_4$-fractionated enzyme)

Dialyze against distilled water through collodion membrane for 24 hrs.

Dialyzed enzyme B

TABLE VII (2)

Experimental No.	Enzyme	Volume	Total protein	Specific activity	Yield
		ml.	*mg.*		%
I	Crude extract	145	2827.5	0.43	100.0
	Heat-treated enzyme	130	461.5	1.55	59.1
	Isoelectrically precipitated enzyme	50	98.0	5.10	41.1
	Gel-treated enzyme	30	27.0	11.56	26.6
	$(NH_4)_2SO_4$-fractionated enzyme	25	16.5	1.70	
				(Cd^{++}, GSH) 10.40	14.1
	Dialyzed enzyme B	34	13.6	0.00	
				(Cd^{++}, GSH) 19.68	22.0
II	Crude extract	190	2773.1	0.39	100.0
	Heat-treated enzyme	171	379.2	1.63	57.3
	Isoelectrically precipitated enzyme	66	84.0	5.15	40.1
	Gel-treated enzyme	40	28.4	10.71	28.2
	$(NH_4)_2SO_4$-fractionated enzyme	30	13.5	1.23	
				(Cd^{++}, GSH) 9.87	12.5
	Dialyzed enzyme B	42	11.7	0.00	
				(Cd^{++}, GSH) 17.62	19.1

Experimental conditions: enzyme solution at each step of purification, pH of which was adjusted to 8.7, 1.0 ml.; 5×10^{-2} *M* L-histidine, 0.2 ml.; 0.2 *M* TRIS buffer (pH 8.7), 0.5 ml.; 3.3×10^{-3} *M* $CdSO_4$, 0.2 ml.; 10^{-1} *M* GSH, 0.04 ml.; final volume, 2.0 ml. with adequate amounts of distilled water. ($CdSO_4$ and GSH were added as indicated.)

After incubation at 37° for 1 hour, 0.2 ml. of 50 per cent trichloracetic acid was added to stop the reaction. Aliquots of the centrifuged supernatants were used for the estimation of L-histidine.

TABLE VIII

Experimental No.	Additions	L-Histidine decomposed	Ammonia liberated	Urocanic acid formed
		μM	μM	μM
I	None	0.00	0.00	0.00
	Cd^{++}	2.20	2.15	2.02
	Folic acid	0.00	0.00	0.00
	GSH	0.57	0.56	0.54
	Cd^{++} and folic acid	2.25	2.10	2.00
	Cd^{++} and GSH	3.32	3.30	3.10
	Cd^{++} and thioglycollate	3.34	3.33	3.21
	Cd^{++} and cysteine	2.85	2.80	2.71
	Cd^{++}, GSH and folic acid	3.31	3.32	3.20
II	None	0.00	0.00	0.00
	Cd^{++}	2.52	2.38	2.19
	Folic acid	0.00	0.00	0.00
	GSH	0.39	0.51	0.40
	Cd^{++} and GSH	3.06	2.97	3.14
	Cd^{++}, GSH and folic acid	3.10	3.02	3.11

Experimental conditions: Dialyzed enzyme B (pH adjusted to 8.7 with 0.1 *N* NaOH), 2.0 ml.; 5×10^{-2} *M* L-histidine, 0.4 ml.; 0.2 *M* TRIS buffer (pH 8.7), 1.0 ml.; 6.6×10^{-3} *M* $CdSO_4$, 0.2 ml.; 4×10^{-3} *M* GSH, thioglycollate or cysteine, 0.2 ml.; 1 per cent solution of folic acid, 0.1 ml.; final volume, 4.0 ml. with adequate amounts of distilled water.

After incubation at 37° for 1 hour in the main chamber of Thunberg tube, 0.6 ml. of 40 per cent trichloracetic acid was tipped in from the side chamber to stop the reaction and to fix ammonia. Aliquots of the centrifuged supernatants were subjected to the estimation of L-histidine, ammonia and urocanic acid.

Michaelis constant (Km) of the enzymatic reaction was about 1.2×10^{-3} *mole/lit.* as calculated from the data presented in Table IX. This value agreed with the one obtained by Walker and Schmidt (*7*) who

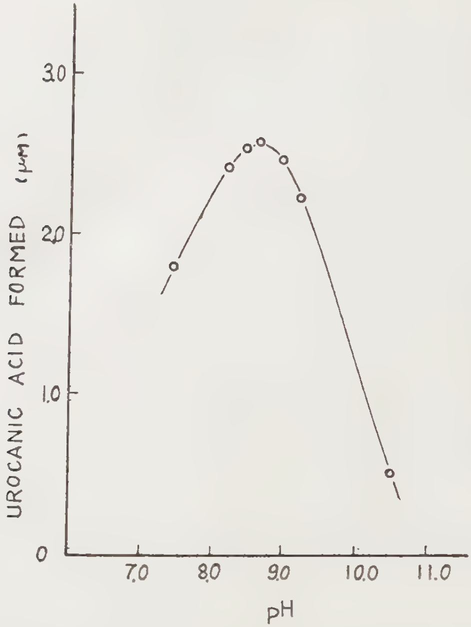


Fig. 2. Experimental conditions: Dialyzed enzyme B (pH adjusted to 8.7 with 0.1N NaOH), 1.0 ml.; 0.1M L-histidine, 0.05 ml.; 0.2M borate-sodium carbonate buffer of varying pH, 1.0 ml.; 0.01M $CdSO_4$, 0.08 ml.; 0.1M thioglycollate, 0.05 ml.; final volume, 2.5 ml. with adequate amounts of distilled water.

After incubation at 37° for 2 hours, 0.5 ml. of 30 per cent trichloracetic acid was added to stop the reaction. Aliquots of the centrifuged supernatants, diluted properly, were subjected to the spectrophotometric estimation of urocanic acid.

employed the enzyme, so called histidase, which was associated with the enzyme system capable of decomposing urocanic acid.

DISCUSSION

Lately, there appeared the reports (*8*, *9*) which suggested the participation of some metallic ion in the activity of L-histidine deaminase. As stated at the beginning of this paper, Ichihara, Uchida, *et al.* held an opinion that three factors, cobalt ion, folic acid and GSH, cooperate as the co-enzyme of this enzyme.

Tht results obtained by us indicate that folic acid was without effect and that cadmium ion alone activated the enzyme, whereas the

TABLE IX

Experimental No.	Substrate concentration	Optical density
	M	
I	1/100	0.297
	1/200	0.293
	1/400	0.260
	1/600	0.216
	1/700	0.200
	1/800	0.196
	1/1000	0.170
	1/1600	0.109
II	1/50	0.172
	1/100	0.166
	1/150	0.160
	1/200	0.153
	1/300	0.134
	1/800	0.094
	1/1600	0.054

Experimental conditions: Dialyzed enzyme B (pH adjusted to 8.7 with 0.1 N NaOH), 1.0 ml., L-histidine in varied concentrations; 0.2 M borate-sodium carbonate buffer (pH 8.7), 0.6 ml.; 6.6×10^{-3} M $CdSO_4$, 0.1 ml.; 4×10^{-2} M thioglycollate, 0.1 ml.; final volume, 2.0 ml. with adequate amounts of distilled water.

After incubation at 37° for 30 minutes, 0.2 ml. of 50 per cent trichloracetic acid was added to stop the reaction. With aliquots of the centrifuged supernatants, diluted 30 folds with distilled water, urocanic acid formed was estimated spectrophotometrically at 265 mμ.

other metallic ions including cobalt were ineffective. Further it was also observed that this enzyme was activated not only by GSH but also unspecifically by other SH-compounds such as thioglycollate or cystein.

These discordances between the results obtained by Ichihara *et al.* and these by us might well be due to the difference in the experimental animals employed; rabbit was used by Ichihara *et al.* and guinea pig by us.

Anyway, it may be stated that L-histidine deaminase is a SH-protein which requires cadmium ion as a co-factor so far as the enzyme from guinea pig liver is concerned.

The authors (*10*, *11*) found that formyl-L-glutamic deformylase which catalyzes the conversion of formyl-L-glutamic acid, an intermediate of L-histidine degradation, to L-glutamic and formic acid was activated by various metallic ions especially by cadmium. It is interesting that among the enzymes concerning L-histidine degradation, at least two enzymes, L-histidine deaminase and formyl-L-glutamic deformylase, require cadmium ion for their activity.

SUMMARY

1. L-Histidine deaminase was isolated from guinea pig liver and purified about forty folds.

2. This enzyme was strongly inhibited by metal- and SH-binders.

3. The enzyme inactivated through dialysis restored its activity by supplementing cadmium ion together with a SH-compound such as GSH, thioglycollate or cysteine.

4. pH optimum of the enzyme was found at 8.7 and Km of the enzymatic reaction was about 1.2×10^{-3} *mole/lit.*

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OXALIC ACID DECARBOXYLASE, A NEW ENZYME FROM THE MYCELIUM OF WOOD DESTROYING FUNGI.

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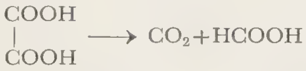
The present author (*1*, *2*) has earlier shown that the wood destroying fungi known as the " brown-rot fungi " are characterized by an abundant accumulation of free oxalic acid in the culture medium (whose pH is below *ca* 5.5), a property not shared by most of the species belonging to the group of " white-rot fungi ".[*]

A striking difference was found to exist between the oxalic acid-non-accumulating form (white-rot) and the oxalic acid-accumulating form (brown-rot). The former has the ability to decompose oxalic acid, while the latter was always found to be devoid of this ability, as far as the manometric detection of the activity was concerned. The decomposition of the acid was shown to be due to the presence in the mycelium of a new enzyme (*4*, *5*) which specifically effected decarboxylation of oxalic acid.



$$\begin{array}{l} COOH \\ | \\ COOH \end{array} \longrightarrow CO_2+HCOOH$$

It is the purpose of the present paper to name and describe the said enzyme, " oxalic acid decarboxylase ", in more detail.

EXPERIMENTAL

Culture—The mycelium of wood destroying fugi[**] was cultured in 300-ml. Erlenmeyer flasks at 25–27° for 25 days on the surface of a liquid medium of the following composition: Glucose 5 per cent, peptone 1 per cent, KH_2 PO_4 0.05 per cent, K_2HPO_4 0.05 per cent, $MgSO_4$ $7H_2O$ 0.05 per cent, KCl 0.05 per cent, and $FeSO_4$ a trace, dissolved in distilled water (pH at the beginning of culture; 5.0–5.2).

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[*] When the culture medium was made alkaline, as by the addition of calcium carbonate to the medium to fix the acid, the production of oxalates was detected also in cultures of some species of white-rot fungi, a characteristic commonly observed among molds in general (*3*).

[**] Strains preserved in the collection of Government Forestry Experimental Station, Tokyo.

Estimation of Enzymatic Activity—The activity of mycelium was estimated by measuring in a Warburg manometer at 30°, the amount of carbon dioxide produced by the decomposition of oxalic acid. Each manometer cup contained 1.3 ml. of a reaction mixture consisting of 1/130 *M* oxalic acid, 1/10 *M* Na citrate-HCl buffer (pH 2.7, unless otherwise noted) and a suspension of mycelium finely ground in a mortar.

RESULTS

Decomposition of Oxalic Acid by the Mycelium of Wood Destroying Fungi—Results of comparative studies on the ability to accumulate oxalic acid

TABLE I

Accumulation of Free Oxalic Acid in the Culture Media (Acidic), vis-a-vis the Decomposition of the Acid by the Mycelium: 19 Species of Wood-destroying Fungi

Fungus	Type of rot	Accumulation of free oxalic acid in culture medium	Decomposition of added oxalic acid by ground mycelium (10 mg. drg weight)
Armillaria mellea	white	—	+
Armillaria japonica	white	—	+
Collyvia veltipes	white	—	+
Coriolus hirsutus	white	—	+
Coriolus versicolor	white	—	+
Fomitopsis pinicola	brown	+	—
Fomitopsis rhodophaea	white	—	+
Lenzites betulina	white	—	+
Phellinus Hartigii	white	—	+
Pholiota adiposa	white	—	+
Pholiota mutabilis	white	—	+
Piptoporus betulinus	brown	+	—
Poria vaporaria	brown	+	—
Shizophillum commune	white	—	+
Stereum fructolosum	brown	+	—
Trametes albida	brown	+	—
Trametes Dickinsii	brown	+	—
Trametes sanginea	white	—	+
Tyromyces sambuceus	brown	+	—

during the course of culture and the ability to decompose the acid added to the medium* (by manometric measurement) are presented in Table I.

TABLE II

Influence of Cultural Age on the Intensity of Oxalic Acid Decomposition by the Mycelium
(10 mg. dry weight For determination of activity; see text)

Days cultured	*Collyvia veltipes*		*Coriolus hirsutus*		*Poria vaporaia*		*Fomitopsis pinicola*	
	pH of culture medium	CO_2 evolved mm.^{3}/30 mins.	pH of culture medium	CO_2 evolved mm.^{3}/30 mins.	pH of culture medium	CO_2 evolved mm.^{3}/30 mins.	pH of media	CO_2 evolved mm.^{3}/30 mins.
15	5.0	1020	5.0	38	1.2	0	—	—
20	4.4	1230	4.8	28	1.2	0	1.2	0
25	4.4	1320	4.4	27	1.2	0	1.2	0
30	4.6	1440	4.6	31	1.2	0	1.2	0
40	5.4	840	4.6	29	—	—	—	—

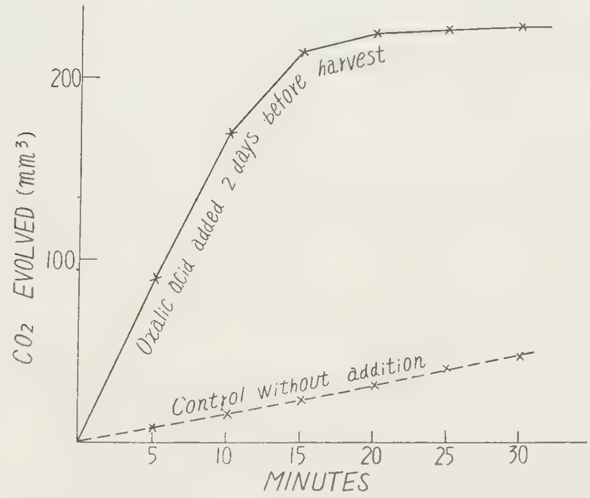


Fig. 1. Influence of the addition of oxalic acid to the culture medium on the oxalic acid-decarboxylase activity of the mycelium of *Ccriolus hirsutus*. (Weight of mycelium 5.5 mg. manometer cup) (Conditions for the determination of activity; see text.)

The coincidence of non-accumulation of oxalic acid in the culture with decomposition of the acid added in the white-rot forms (11 species) was striking, while non-decomposition of oxalic acid was confirmed in the brown-rot forms (7 species), where abundant yield of free oxalic acid in the culture medium was always found.

Influence of Cultural Age on the Intensity of Oxalic Acid Decomposition by the Mycelium—The mycelium of four species of wood destroying fungi, two (*Collyvia veltipes* and *Coriolus hirstutus*) belonging to the whit-rot group, and the other two (*Poria vaporaria* and *Fomitopsis pinicola*) to the brown-rot group, were cultured and harvested at the end of 15, 25, 30, and 40 days of culture period. No significant change was observed except in the case of *Collyvia veltipes*, where a slight decrease in the activity was observed at the last stage (40th day of culture), probably due to the change in pH of the medium during the culture.

TABLE III

Activation of Oxalic Acid Decarboxylase by the Addition of Acids to the Culture Media of Coriolus hirsutus

(For conditions for the determination of activity; see text. (10 mg. mycelium) dry weight). (The decarboxylase activity was estimated 2 days after addition of acids to the 30-day-old culture of Coriolus hirsutus.)

Acid added	Concentration of acid added	pH of the medium (after 2 days)	Kalkessig reaction	CO_2 evolved (mm.^{3} per 5 mins.)
None		5.2	–	5
Oxalic acid	0.025 %	5.2	–	60
	0.05	5.0	–	67
	0.1	5.0	–	122
	0.2	5.0	–	203
	0.3	4.4	±	185
	0.4	2.2	+	37
Glycolic acid	0.3	3.2		540
Malonic acid	0.2	3.0		158
Citric acid	0.2	3.6		59
Hydrochloric acid	0.1	2.8		403
	0.06	3.2		277

Effect of Addition of Acids to the Culture Medium—Oxalic acid (2.5 mg. per ml. culture medium) was added to 25-day-old culture of Coriolus hirsutus. After 2 days, the mycelium was harvested and tested for the activity. A typical result is shown in Fig. 1, indicating an increased activity of the treated mycelium beyond that of the control, to which no additional oxalic acid had been given. Addition of other acids (*e.g.*, hydrochloric acid) also had an ameliorating effect on the enzymatic activity of the mycelium (see Table III), suggesting the favorable effect of higher hydrogen ion concentration on the formation of the enzyme in the mycelium.

Changes in the enzymatic activity of the mycelium treated as above were followed and the results were plotted as a function of time (hours) after the addition of hydrochloric acid to the culture medium (Fig. 2).

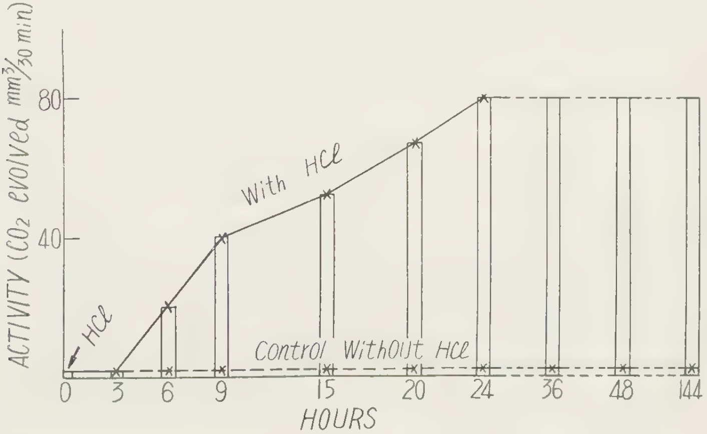


Fig. 2. Oxalic acid-decarboxylase activity of the mycelium of *Coriolus hirsutus*, as influenced by the addition of hydrochloric acid to the culture medium. Enzymatic activity is expressed in CO$_2$ (mm.^{3}) evolved during the first 30 minutes by the mycelium (1 mg. dry weight).

(Condition for the determination of activity; see text.)

Full activation of the mycelium was reached at 24 hours after the addition of the acid, and the attained level was maintained further on. Optimum pH of the medium effecting the highest level of activity of the harvested mycelium was found to be about 3.0 (Fig. 3).

Isolation of the Enzyme from the Mycelium—The mycelium of *Coriolus hirsutus* and *Collyvia veltipes* cultivated for about one month and "fortified"

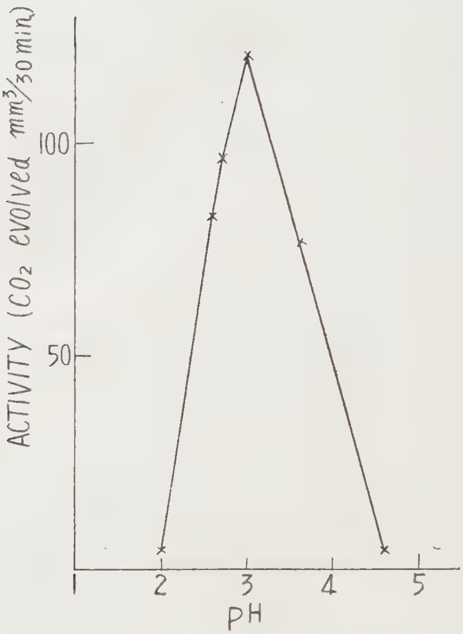


Fig. 3. Influence of pH of culture medium on the oxalic acid-decarboxylase activity of the mycelium (*Coriolus hirsutus*). (Varying amounts of hydrochloric acid added 2 days before harvest of the mycelium to adjust the pH of the culture media.)

Enzymatic activity expressed in CO_2 (mm.^{3}) evolved, during the first 30 minutes by the mycelium (1 mg. dry weight). (Condition for the determination of activity; see text.)

by the addition of hydrochloric acid for 2 further days as described above, was ground in a mortar, the resultant paste was extracted with distilled water, the mass of mycelium debris was discarded, and to the clear supernatant of centrifugation were added 2–3 volumes of cold acetone. The precipitate thus formed was collected and dried in vacuum and redissolved in distilled water, insoluble debris being discarded by centrifugation. The clear solution was dialyzed for 2 days in an ice-chest against a large quantity of distilled water, renewed from time to time. After centrifuging the liquid remaining in the cellophane sack and discarding the insoluble material, a clear solution with some tinge of yellow was obtained, which retained the enzymatic activity in quesion.

Further investigations on the mode of action of the enzyme were carried out with this preparation, the results from which are described in the following.

Decomposition of Oxalic Acid in the Presence of the Enzyme—The evolution of carbon dioxide was followed manometrically (see Fig. 4). On completion of the reaction when the production of CO_2 was no longer observed, no oxalic acid, remained in the reaction mixture, as estimated by the "Kalk-essing" reaction. The total amount of CO_2 evolved was in fair agreement with the value calculated by assuming 1 mole CO_2 to evolve per mole of oxalic acid consumed. One of the typical results may be exemplified as follows:

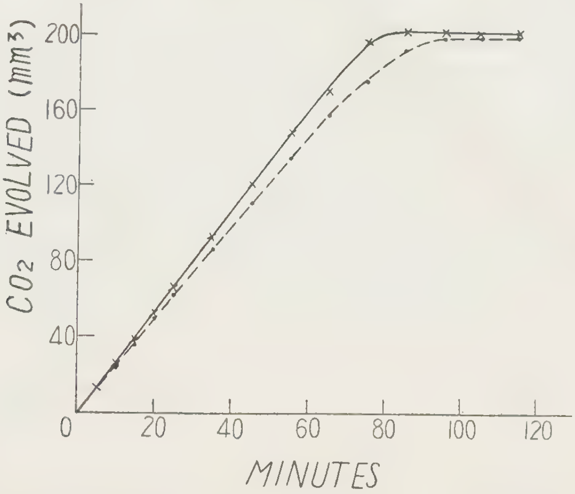
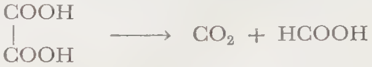


Fig. 4. Decomposition of oxalic acid by the cell-free preparation of oxalic acid-decarboxylase from the mycelium of *Collybia veltipes*.
(×——×) *M*/4 Na citrate-HCl buffer (pH 2.7)
(•- - - -•) without buffer
(oxalic acid 1.26 mg. and enzyme 0.17 mg. dry weight, total volume 1.3 ml.; see text.)

Reaction mixture:	*M*/30 oxalic acid, 0.3 ml. *M*/4 Na citrate-HCl buffer (pH 2.7), 0.7 ml. enzyme solution, 0.3 ml.
Total amount of	CO_2 evolved: 230.3 mm.^{3} CO_2 calcd.: 224.0 mm.^{3}

Though the manometric determinations were run under aerobic conditions (air), no perceptible absorption of molecular oxygen was recognized.

Identification and Estimation of Formic Acid—The nearest conjecture which is in harmony with the above results is an idea of decarboxylation of oxalic acid into carbon dioxide and formic acid.



$$\begin{array}{c}\mathrm{COOH}\\ | \\ \mathrm{COOH}\end{array} \longrightarrow \mathrm{CO_2} + \mathrm{HCOOH}$$

Hence, an experiment was planned for the quantitative determination of total formic acid produced as the result of the reaction. Six ml. of enxyme solution were added to 60 ml. of $M/60$ oxalic acid, the mixture was incubated for 14 hours at 28°C, and then steam distilled. Formic acid in the distillate was identified by the melting point of its *p*-bromophenacyl ester (m.p. 135°), and determined as usual by weighing the amount of calomel formed by the reaction of the acid with mercuric chloride: 13.67 mg. formic acid recovered in the distillate, to account for 90 per cent of the theoretical value.

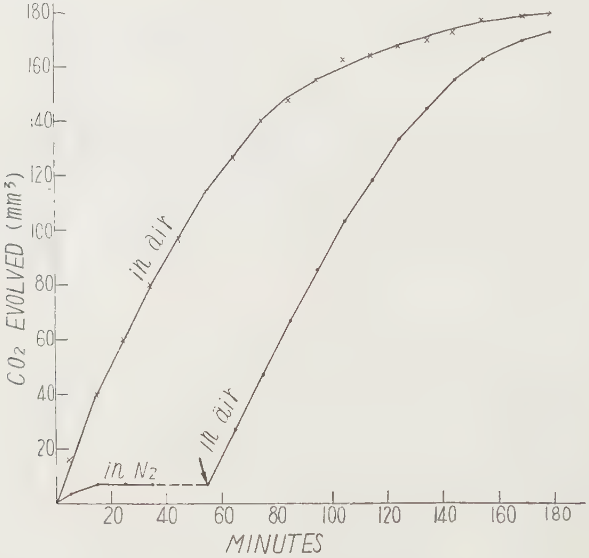


Fig. 5. Influence of atmospheric oxygen on the activity of oxalic acid-decarboxylase. (*Collyvia veltipes*) (enzyme 0.03 mg. N) (Condition for the determination of activity; see text.)

Activating Effect of Molecular Oxygen on the Enzymatic Decomposition of Oxalic Acid—The presence of molecular oxygen seems to be a requisite condition for the activity of the enzyme, though it is neither involved in the final balance sheet (see above), nor is any actual mode of its intervention apparent in the scheme of the reaction.

The effect of aero- or anaerobiosis seems to be completely reversible: An enzyme solution in the manometer cup which had previously been manoeuvred in an atmosphere of nitrogen for about 40 minutes, to prove the complete absence of the reaction, recovered its full activity when air was introduced into the gaseous phase (Fig. 5). Detailed investions on the mechanism of the action of oxygen was postponed to further studies, and all experiments in the present work were carried out in an aerobic condition (air).

Heat Stability and Influence of Hydrogen Ion Concentration—The enzyme is relatively stable to high acidity and tolerated a heating as high as 70°.

Temperature of heating* (°C/*10 minutes*)	100°	90°	80°	70°
Loss of activity (%)	100	100–64	40–20	0

The optimal pH of the reaction was found to be about 2.5–3.0. At a pH higher than 5.5 or lower than 1.5, the enzyme was practically inactive (Fig. 6).

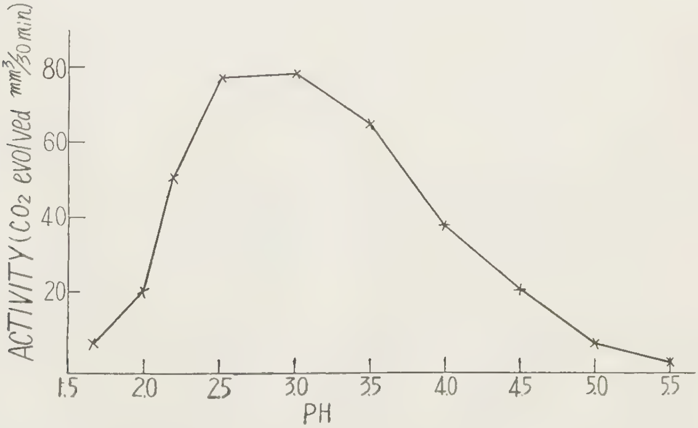


Fig. 6. Influence of pH of the reaction medium on the activity of oxalic acid decarboxylase (*Collyvia veltipes*).

(*N*/2 Na citrate-HCl buffer, oxalic acid 1.26 mg., and enzyme 0.17 mg.)

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* Acetone treated enzyme

Affinity towards the Substrate—Fig. 7 represents the velocity of the reaction as a function of the substrate concentration. The half-maximal velocity was measured at the point corresponding to the concentration of oxalic acid, 10–3.89 *M*. Hence, the Michaelis constant K_M is,

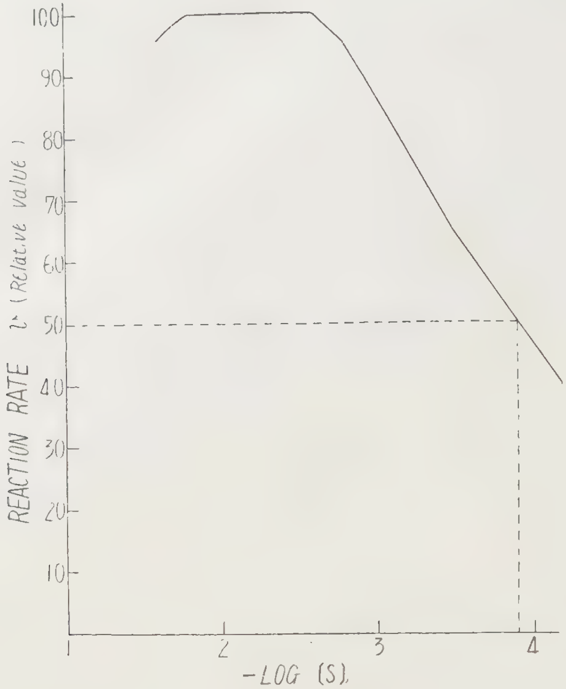

Fig. 7. Influence of substrate concentration on the velocity of oxalic acid decomposition (*Collyvia veltipes*).

V : reaction rate (relative value in percent of maximum value).

S : concentration of oxalic acid (*mol./lit.*)

K_M=0.000125 *mol./lit.*, (pH 2.7, 30°)

$K_M=1.25\ 10^{-4}$ *mol./lit.* (pH 2.7, Na citrate-HCL buffer, 30°)

Specifity of the Enzyme—So far as the present knowledge is concerned, oxalic acid represents the sole substrate to be attacked by the enzyme, pyruvic*, malonic, succinic, glycolic, citric, malic, oxalacetic*, and formic acids, all being inert in this respect.

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* With and without the addition of cocarboxylase and Mg++.

TABLE IV

Inhibition and Acceleration of Oxalic Acid Decarboxylase

(A) The chemicals added 15–30 minutes before the addition of the substrate.

(B) The chemicals added 10–20 minutes after the addition of the substrate.

Concentration *M* Chemicals	10^{-3}	10^{-4}	10^{-5}
KH_2PO_2	(A): none, (B): none	(A): none, (B): none	—
Hydroquinone	(A) inhibition∼** acceleration (B) 70–100% acceleration	(A) inhibition∼** acceleration (B) 70–100% acceleration	(A) acceleration (B) 50–70% acceleration
Ascorbic acid	(A), (B) as above	”	”
Pyrocatechol	”	”	”
$FeSO_4$	(A) inhibition∼** acceleration (B) 32% acceleration	(A) — (B) 6% acceleration	—
$Fe_2(SO_4)_3$	(A): none, (B): none	(A): none, (B): none	—
Glutathione	(A), (B) as above	”	—
Cysteine	”	”	—
Cystine	”	”	—
α, α'-Dipyridyl	”	”	—
Benzensulfonic acid	”	”	—
Monoidoacetic acid	(A) 80% inhibition (B) 65% inhibition	(A) — (B) 20% inhibition	—
Monofluoroacetic acid	(A): none, (B): none	—	—
NaF	”	(A): none, (B): none	—
Sodium arsenate	(A), (B) as above	”	—
Sodium arsenite	”	”	—
KCN	”	”	—
$CuSO_4$	(A) 84% inhibition (B): none	(A) 60% inhibition (B): none	—

TABLE IV (continued)

Concentration M Chemicals	10^{-3}	10^{-4}	10^{-5}
$HgCl_2$	(A): none, (B): none	(A): none, (B): none	—
$MnCl_2$	”	”	—
$MgSO_4$	”	”	—
$CoCl_2$	”	”	—
EDTA	”	”	—
PCMB	”	”	—
Malonic acid	”	”	—
Methylene blue	”	”	—
Benzoquinone	(A) 30–50% inhibit (B): none	—	—
H_2O_2	(A) 70% inhibition (B) 53% inhibition	(A) 40% inhibition (B) 40% inhibition	—

** At first inhibition occurs, and then acceleration follows.

Activators and Inhibitors of the reaction—The effect of chemicals on the enzymatic decomposition of oxalic acid is summarized in Table IV. Addition of H_2O_2, $CuSO_4$ monoiodoacetic acid ($10^{-3}M$) to the enzyme solution (before the addition of the substrate) resulted in a more or less marked inhibition of the reaction. Addition of one of the following substances (also in a concentration of $10^{-3}M$) was found to be without effect: Glutathione, cysteine, cystine, α, α'-dipyridyl, benzenesulfonic acid, monofluoroacetic acid, malonic acid, methylen blue, EDTA, PCMB, KH_2PO_2, $Fe_2(SO_4)_3$, NaF, Na arsenate, Na arsenite, KCN, $HgCl_2$, $MnCl_2$, $MgSO_4$, and $CoCl_2$.

Some chemicals exhibited an accelerating effect when added to the system after or together with the substrate (hydroquinone, ascorbic acid, pyrocatechol, and $FeSO_4$). It must be mentioned here that the addition of these substances before the substrate was added to the enzyme solution caused at first a temporary inhibition of the start of the reaction. The inhibition in this case, however, was a fugitive one, to be gradually and finally replaced by the accelerating effect, common to this group of substances. In more dilute concentrations (*e.g.* $10^{-5}M$) they exhibited only the aeliorating site of their influence, the evolution of CO_2 in manometer cups with the chemicals being more rapid from the beginning.

The inhibitory effect of monoiodoacetic acid and H_2O_2 seemed to be independent of the order in which the substrate and the chemical is given to the enzymatic system.

Effect of Mycelium Extract—With crude enzyme (mycelium extract), no accelerating effect of boiled mycelium extract was perceptible (Fig. 8: A), but when purified preparations (dialyzed solution of the crude enzyme or acetone-treated preparations) were used, addition of a boiled mycelium extract exhibited a definite and marked increase in the velocity of the reaction (Fig. 8: B), suggesting the existence of a cofactor (or

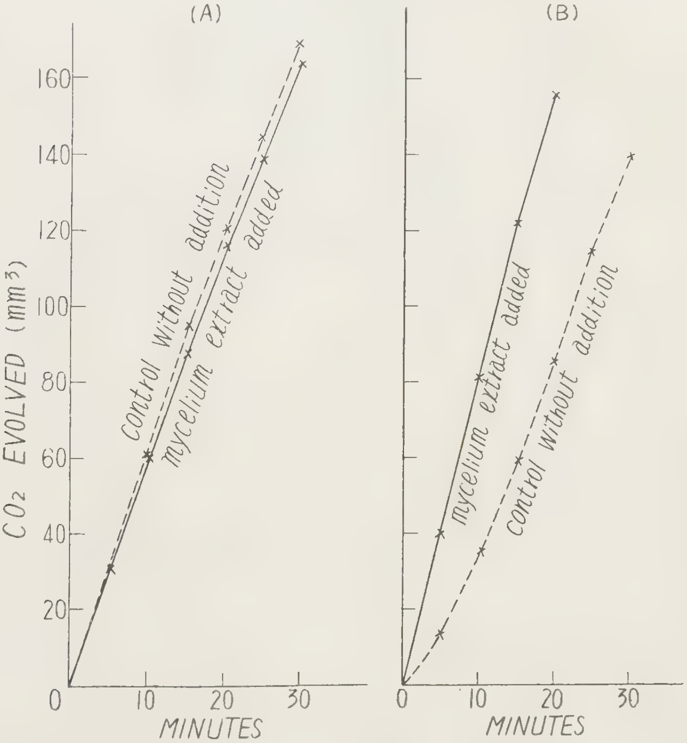

Fig. 8. Effect of boiled mycelium extract on the activity of crude and/or purified preparation (oxalic acid-decarboxylase of *Collyvia veltipes*.)

(A); crude enzyme (0.2 mg.N) (B); acetone-treated enzyme (0.06 mg.N)

(Condition for the determination of the activity; see text.)

-factors) supplementing the enzyme.

Dialysis of the solution of crude enzyme resulted in a more or less marked detelioration of the activity, to be recovered fully on adding a sufficient amount of boiled mycelium extract (Fig. 9). Dialysate of the boiled mycelium extract showed the same complementary action.

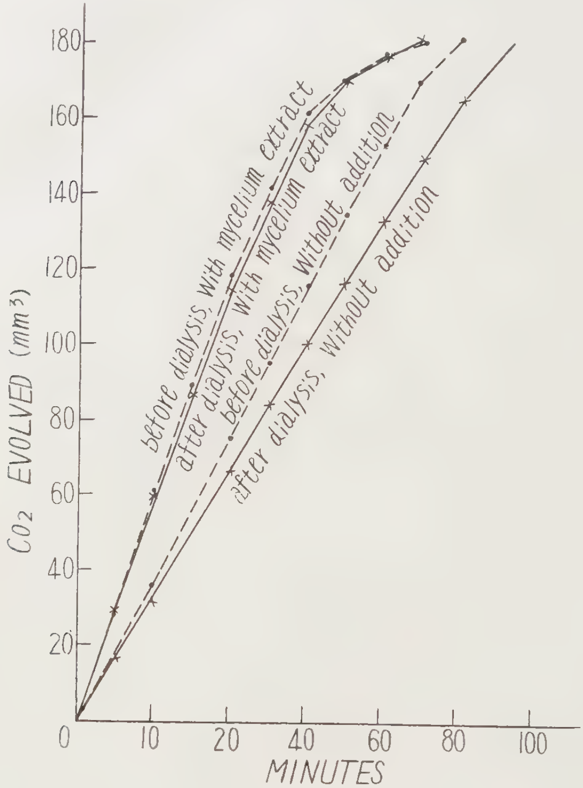

Fig. 9. The effect of boiled mycelium extract on the decarboxylase of oxalic acid by the enzyme preparation before and after dialysis.* (Acetone treated enzyme; 0.05 mg. N) (before dialysis)

Neither the residue of the dialysis, nor the ash of the mycelium extract showed such action (see Fig. 10).

The action of accelerators, also, seems to be dependent on the

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* Correction was made for the value change of the enzyme solution due to dialysis.

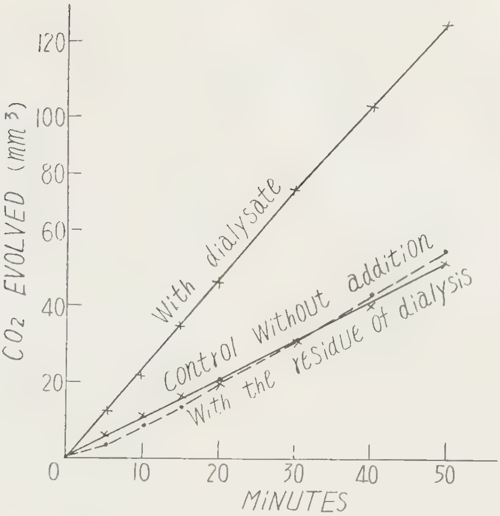

Fig. 10. Effect of the dialysate and of the residue of dialysis of boiled mycelium extract on the oxalic acid-decarboxylase. (acetone-treated enzyme; 0.01 mg.N)

(Condition for the determination of activity; see text.)

existence (or concentration) of the supplementing factor stated just above (Fig 11). An accelerating action by pyrocatechol (added in the middle of the reaction: see Fig. 11) was only realizable with a purified enzymes. The activating effect of pyrocatechol and the boiled mycelium extract seemed to be of complementary nature; the elevated level of activity after the addition of the boiled mycelium extract was not changed by further addition of pyrocatechol (Fig. 12).

Cocarboxylase (0.5 mg. in 1 ml. of the reaction mixture) had no effect on the activity of the enzyme (crude or purified, and with or without Mg^{++}).

DISCUSSION

The ability to decompose oxalic acid, whether aerobically or anaerobically, is an activity shared by various representatives of the vegetable kingdom; higher plants, mosses, molds, and bacteria. The aerobic oxidation of the acid in the course of respiration in various bacteria was studied by Bassalik (*6*), Bhat, and Barker (*7*) and recently by

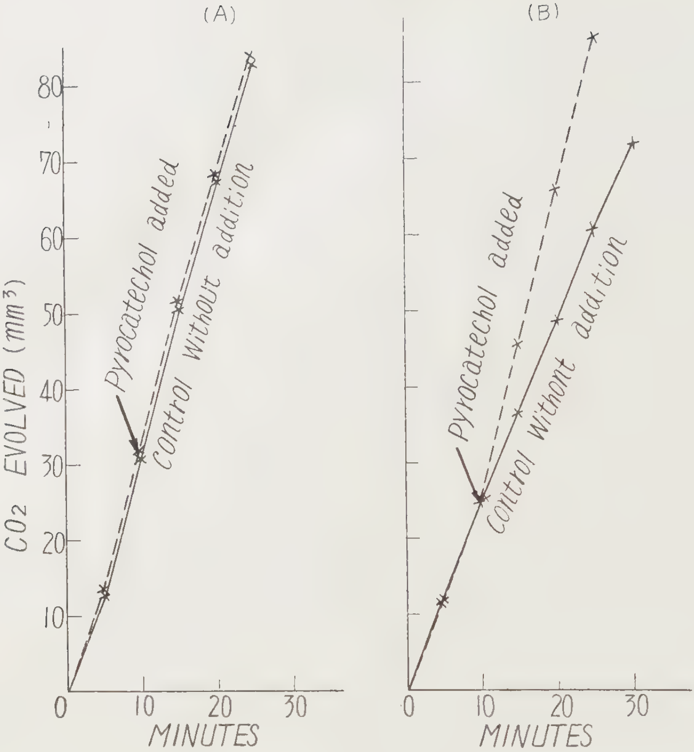

Fig. 11. Effect of pyrocatechol on the oxalic acid decarboxylase of *Collyvia veltipes*.

(A); crude enzyme (0.1 mg.N) (B); acetone-treated enzyme (0.03 mg.N)

Takamiya and Kaneda (*8*). There are numerous informations on the accumulation and disappearance of oxalic acid in the culture of mold species*.

In 1934, Chrzasz and Zakomory (*9*) reported that formic acid is produced from oxalic acid during the growth of penicillium, aspergillus, and rhizopus. The appearance and disappearance of oxalic acid in the culture medium of *Aspergillus niger* was studied by Sakaguchi and Baba (*10*). In a series of his works on the acid formation by

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* For the general review of the matter, see Foster, J. W., Chemical Activity of Fungi (1949) (*3*).

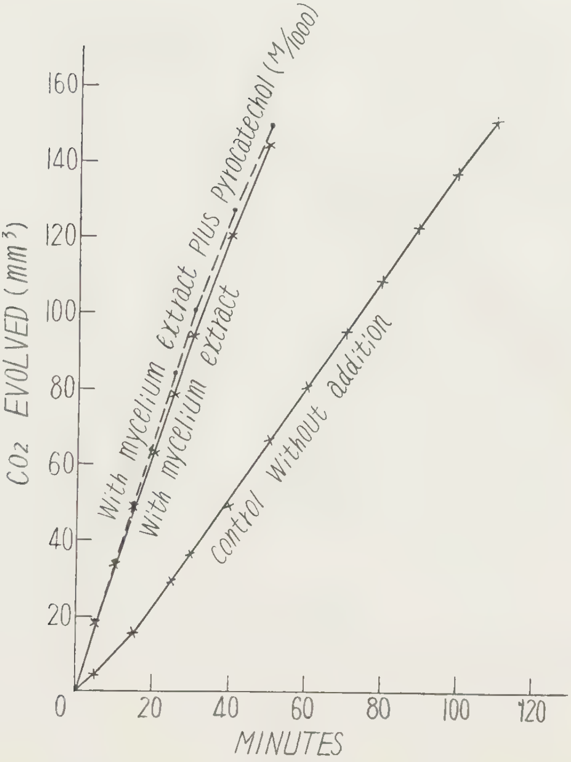


Fig. 12. The effect of boiled mycelium extract and pyrocatechol on the oxalic acid decarboxylase of *Collyvia veltipes*. (enzyme; 0.01 mg.N)

(Condition for the determination of activity; see text.)

molds, F. F. Nord (*11*) mentioned that a wood destroying fungus, *Merulius niveus*, could utilize free oxalic acid in the culture solution, but not its sodium salt. The decomposition of oxalic acid into formic acid and carbon dioxide by the mycelium of *Shizophillum commune* (white-rot fungi) was recently reported by Shibamoto and Fukuzumi (*12*).

The results of the writer's comparative studies on the production (in the culture medium) and the decomposition (by the mycelium) of oxalic acid by white-rot and brown-rot fungi, seem to support the writer's assumption that the absence of the acid in the culture of white-rotters is due to the presence in these organisms of an enzyme which destroys the acid. The accumulation of the acid in the culture of the brown-rotters, all of which lack the enzyme, furnishes support in favor of the theory. As for the enzymes which habe hitherto been isolated, they all belong to the category of oxidases or dehydrogenases, oxidizing the substrate in the presence of molecular oxygen or other suitable acceptors of hydrogen (*13*). Franke and Hasse (*14*) and Niekerk (*15*) extracted from mosses an enzyme oxidizing oxalic acid into carbon dioxide and hydrogen peroxide. Nagahisa and Hattori (*16*) succeeded in isolating oxal-oxidase from the grana of the chloroplast of higher plants. The enzyme isolated and partially purified in the present work belongs to another group of biocatalyst; the decarboxylase. It deffers from all the known representatives of this group in that it is specific to the decarboxylation of oxalic acid. Hence it will be named here the "oxalic acid-decarboxylase". Cocarboxylase known as the common co-factor complementary to most decarboxylating enzymes seems to have no role in the reaction of this enzyme. Results of the present series of experiments concerning the effect of dialysis or acetone treatment, and of the addition of boiled mycelium extract on the activity of the enzyme suggest the complex nature of the enzyme system. Detailed investigations on the nature of the complementary principle in question, together with (the action of) inhibitors and accelerators of the reaction are in progress.

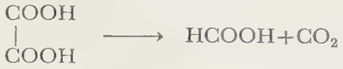
SUMMARY

1. Comparative studies on the production (in the culture medium) and the decomposition (by the mycelium) of oxalic acid by white-rot and brown-rot fungi were carried out and it was found that the former group of fungi is characterized by their inability to produce free oxalic acid in the medium together with the ability of the mycelium in decomposing the acid. The contrary was the case with the group of the brown-rot fungi.

2. The influence of cultural age and the effect of the addition of acids to the culture medium on the intensity of the enzymatic activity of the mycelium were studied.

3. A soluble enzyme responsible for the decomposition of the acid was isolated from the mycelium of *Coriolus hirsutus* and *Collyvia veltipes* (white-rot fungi) and the procedure for partial purification of the enzyme was described.

4. The enzyme was shown to be strictly specific for the decarboxylation of oxalic acid.



$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} \longrightarrow HCOOH+CO_2$$

It differed from all the known enzymes, affecting the decomposition of oxalic acid or the decarboxylation of other acids. Hence, the name of "oxalic acid decarboxylase" was proposed for the new enzyme.

5. Addition of mycelium extract to a dialyzed solution of the enzyme had an ameliorating effect on the reaction, suggesting the complex nature of the enzyme system.

6. Cocarboxylase had no effect on the reaction of the enzyme.

7. Aerobic condition was shown to be the requisite for the activity of the enzyme, though no consumption of oxygen was ever detected by the manometric measurements.

8. The Michaelis constant of the enzyme, K_M, was found to be 1.25×10^{-4} *mole/lit.* (pH 2.7, Na citrate-HCl-buffer, 30°)

9. Activating and inhibiting effects of chemicals on the enzyme reaction were described.

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BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

I. THE KINDS OF FREE AMINO ACIDS CONCERNED IN THE BIOSYNTHESIS OF THE SILKPROTEIN

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(Received for publication, February 21, 1955)

Numerous studies on protein biosynthesis have been made with the pancreas, the chick embryo, the oviduct, the mammary gland, the silkgland, the tissue slices, bacteria *etc.*, generally speaking, as a material for the study of protein biosynthesis, it is desirable to take an organ in which the protein synthesis is particularly active than any other reactions. Little is known of the kinds of free amino acids used for the biosynthesis of a particular protein. Activities of the silkgland of the silkworm are mostly the synthesis of the silkproteins, taking up precursors in the body fluid to secrete fibroin protein in the posterior division and sericin protein in the middle division (*1*). Therefore we took the silkgland of the 5th stage as a particularly suitable system for our purpose. It is supposed from the study of biosynthesis of milkprotein (*2*), ovalbumin (*3*), *etc.* that the nature of the precursors of the silkprotein biosynthesis are free amino acids, peptides or proteins, present in the body fluid. If the silkprotein is directly derived from free amino acids in the body fluid, free amino acids removing from the body fluid to the silkgland cells would be main amino acid constituents of the silkprotein. If the protein is, on the other hand, derived from other precursors than free amino acids in the body fluid, this relationship would not be recognized.

To clarify the kinds of free amino acids removing intensively to the silkgland cells, it is necessary, as a method, to dam the stream of free amino acids from the body fluid to the silkgland cells in the larval body, and as a suitable system for this purpose, the silkglandectomized larva was used. The silkglands at the end of the 5th stage of the normal larva occupy a great part of the volume of the larval body

(Fig. 1). Therefore, if free amino acids in the body fluid take part in the formation of the silkprotein, it would be expected that the extirpation of the glands at the younger stage exerts notable effect on free amino acid concentration in the body fluid at the end of the 5th stage (*4*). Free amino acid concentrations in the body fluid of the ectomized and normal larvae were determined at the 3rd and the 6th day of the 5th stage. Biosynthesis of the protein in the silkgland cells is remarkably active at the 6th day, but not yet at the 3rd day.

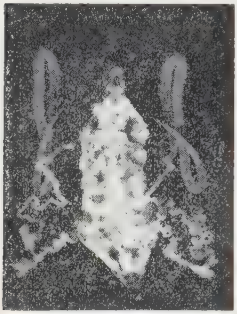

Fig. 1. The Silkglands at the 6th day of the 5th stage (Si 108 (Kyu))

EXPERIMENTAL

Materials were obtained from the silkglandectomized* and normal larvae (Si 108 (Kyu)) at the 3rd and the 6th day of the 5th stage. The procedure to prepare the samples for the microbiological assay was, in principle, made following the method described by Elvehjem *et al.* (*5*) for the assay of rat blood plasma except for the precipitating agent. The precipitation of the proteins in the body fluid was carried out with trichloracetic acid instead of tungstic acid as a precipitating agent, because free amino acids in the body fluid, especially some of the basic amino acids, such as histidine and lysine, are precipitated together with the protein by using tungstic acid according to the procedure of Elvehjem *et al.*, while they are not precipitated by using trichloracetic acid (*6*). 10 ml. of the body fluid which was collected in a tube kept in ice water was added in a bottle containing 90 ml. of 3 per cent trichloracetic acid, and the bottle and contents allowed to stand at room temperature for 30 minutes with intermittent shaking. The precipitates were removed by centrifugation, and the filtrates were heated in a boiling water bath for few minutes. The clear protein-free filtrates were neutralized to pH 6.8 with *N* sodium hydroxide and filled up to 100 ml. with distilled water and stored in a refrigerator under toluene until determinations were carried out.

The microbiological assays on seventeen free amino acids in trichloracetic acid filtrates were carried out by the methods described by Tamura *et al.* (*7*).

RESULTS

Free amino acid concentrations in the body fluid determined by the microbiological method are shown in Table I. In general, the concentration of each free amino acid in the body fluid of the silkworm larvae is strikingly different from those of the silkproteins (Table II)

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* The silkglands were extirpated at the beginning of the 4th stage.

TABLE I

Free Amino Acid Concentrations in the Body Fluid of the Silkworm Larva

L-form

Amino acids	3rd day of 5th instar		6th day of 5th instar		Test organism used*
	Silkgland-ectomized larva	Normal larva	Silkgland-ectomized larva	Normal larva	
	μg./ml.	*μg./ml.*	*μg./ml.*	*μg./ml.*	
Alanine	263	305	160	173	Cit
Arginine	416	356	205	210	M
Aspartic acid	80	60	230	240	M
Glutamic acid	95	90	172	175	S
Glycine	897	297	2710	129	M
Histidine	2172	2199	2536	2805	Cit
Isoleucine	63	75	25	46	M
Leucine	166	153	82	116	M
Lysine	1018	889	860	1042	M
Methionine	84	97	54	78	M
Phenylalanine	71	73	90	96	M
Proline	371	103	674	104	Cit
Serine	656	201	1131	463	M
Threonine	885	314	2097	237	S
Tryptophan	25	32	93	52	M
Tyrosine	394	279	517	202	M
Valine	226	255	205	138	M
Total	7882	5778	11841	6306	

* Cit: *Leuconostoc citrovorum* M: *Leuconostoc mesenteroides*
S: *Streptococcus faecalis*

and the mulberry leaves proteins (*8*), and the values are peculiar to when compared with free amino acid of rat (*9*) and human blood plasma (*10*). The peculiarity of the body fluid is in its far higher concentration of basic amino acids, such as histidine and lysine (*6*). Between the 3rd day normal larvae (when biosynthesis of the silkprotein in the silkgland cell is not active yet) and the 6th day ones (when biosynthesis is remarkably active), no significant difference is observed for each free amino acid, because of dynamic equilibrium in the body

TABLE II

Amino Acid Composition of Fibroin, Sericin and Cocoon Fibre Determined by the Microbiological Method

Amino acids	Fibroin	Sericin	Cocoon fibre (Silkprotein)
	%	%	%
Alanine	28.30	4.00	25.00
Arginine	0.83	4.09	1.65
Aspartic acid	1.60	16.80	5.50
Glutamic acid	1.37	10.65	2.10
Glycine	42.80	8.60	33.60
Histidine	0.35	1.39	0.73
Isoleucine	1.30	0.80	1.45
Leucine	1.00	0.88	0.96
Lysine	0.42	5.84	1.22
Methionine	0.11	—	0.15
Phenylalanine	1.07	0.65	1.11
Proline	0.48	—	0.48
Serine	14.70	30.10	16.00
Threonine	1.12	5.18	3.00
Tryptophan	0.26	—	0.49
Tyrosine	13.75	3.80	12.35
Valine	2.88	1.85	3.16

fluid of free amino acids. At the 3rd day, proline, glycine, serine, threonine and tyrosine concentrations in the body fluid of the ectomized larva are slightly higher than those of the normal larva while other amino acid concentrations are almost the same between both. On the other hand, between the ectomized larva and the normal at the 6th day, the former is far higher than the latter in the concentration of some free amino acids; 20 times at glycine, 9 times at threonine, 6 times at proline, 3 times at serine and twice at tyrosine, and for other amino acids, no significant difference is observed. In addition to this, the quantity of the body fluid in the glandectomized larva is about 1.3 times at the 3rd day, and about twice at the 6th day, as much as that of the normal larvae respectively.

A similar phenomenon is also observed from the results of paper chromatograhy *(11)* carried out on the same four samples. Namely, the body fluid of the ectomized larvae at the 3rd [day, and more remarkably at the 6th day, is different from that of the normal larvae in the amino acid concentration and seems to be especially high for glycine and serine concentration.

These results show that, during biosynthesis of the silkprotein, free amino acids removing intensively from the body fluid to the cell of the silkgland seem to be glycine, threonine, serine, proline and tyrosine.

DISCUSSION

Silkprotein consists of 70–75 per cent of fibroin and 25–30 per cent sericin, and its main constituent amino acids are, as shown in Table II, glycine, alanine, serine and tyrosine. On the other hand, during biosynthesis of the silkprotein, free amino acids removing intensively from the body fluid to the cell of the silkgland seem to be, as is understood from Table I, glycine, threonine, serine, proline and tyrosine, and among these, glycine, serine and tyrosine are in good agreement with main amino acid constituents of the silkprotein. This fact seems to suggest to us that some part of glycine, serine, tyrosine, threonine and proline in the silkprotein are directly derived from free amino acids present in the body fluid. On the contrary, during protein biosynthesis in the silkgland cell, alanine, as it is one of main constituent amino acids of the silkproteins, does not almost remove, in a free form, from the body fluid to the cell of the silkgland. The ratio of glycine to serine or tyrosine in free amino acids removing intensively from the body fluid to the silkgland cell is slightly lower than that in the silkprotein. And threonine in the body fluid removes comparatively in great quantities to the silkgland cell while aspartic acid and valine, as their content in the silkprotein is the same as threonine content in it, do not almost remove to the silkgland cell. These facts seem to suggest to us that the silkprotein is derived not only from particular free amino acids, but also from other precursors in the body fluid. These precursors may be peptides or proteins which remove from the body fluid to the silkgland cell, as they are, or such substances as are convertible to amino acid in the cell.

SUMMARY

1. Present study was carried out to clarify the kinds of amino acids concerned in the silkprotein biosynthesis.

2. During biosynthesis of the silkprotein in the silkgland, glycine, serine, threonine, tyrosine and proline remove intensively, in a free form, from the body fluid to the cell of the silkgland, but it is scarcely the case for free alanine.

3. These facts seem to suggest to us the following. Some part of glycine, serine, tyrosine, threonine and proline in the silkprotein must have their origin in free amino acids in the body fluid, but alanine which is one of the main constituent amino acids of the silkprotein is not derived from free alanine in the body fluid.

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ON THE HEMATIN LINKAGE IN METHEMOGLOBIN

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It has been pretty clearly established through the studies of Wyman *et al.* (*1*, *2*) that in the molecule of oxyhemoglobin the globin moiety is linked to each heme by virtue of two atom-groups which, based on their acid-base characters, were assumed to be imidazoles of histidine. By similar investigations carried out with methemoglobin (MHb^+) the dissociation constant (pK′) of the hematin-linked group has been determined (*3–7*). The present paper deals with the study of the same group in the molecule of cyan-methemoglobin (MHbCN), using two methods of approach; namely:

(i) by measuring spectrophotometrically the dissociation of cyan-methemoglobin, $MHbCN \rightleftarrows MHb^+ + CN^-$, in aqueous solutions of various pH values, and

(ii) by conducting acid-base titrations on MHb^+ and MHbCN.

Preparation of the Material

Methemoglobin—MHb^+ used in these experiments was obtained by the oxidation of the oxyhemoglobin which was prepared from horse blood following Ferry and Green's method (8). Recrystallized oxyhemoglobin dissolved in KCl solution (0.5 *M* for spectrophotometric measurement and 1 *M* for titrimetric experiment) was oxidized with ferricyanide and stored after filtration.

Buffer solutions: Use was made of phthalate buffers for the pH range of 4.5–6.5 and phosphte buffers for the pH range of 6.5–7.5. Experiments in more alkaline pH ranges were carried out using borate buffers.

KCN Solution—The concentration of KCN was determined by Liebig's method. In spectrophotometric experiment, pH of the solution was adjusted to the desired value before each experiment.

Spectrophotometric Measurements

Solutions used in the spectrophotometric measurements were prepared by the following procedure. The stock solution of MHb^+ mentioned above was dialysed against distilled water over-night in an ice-box, and diluted with distilled water. To 2 ml. of the diluted MHb^+ solution, 1 ml. of buffer solution and 1 ml. KCN (diluted to 10^{-2}–10^{-5} *M*) was added, and finally the total volume of the solution was made to 10 ml. by adding distilled water. The ionic strength of the final solution was calculated to be 0.01. The solution was allowed to stand at room temperature for 2 hours prior to optical density measurements, since the initial optical density decreased slowly with time and reached a constant value only after about 2 hours. The optical density measurements of MHb^+ solutions containing various amounts of KCN was conducted using the Beckman DU spectrophotometer. Temperature was maintained constant within 0.2° by the use of a Beckman thermospacer. After measurements of the optical density, the pH value of each sample was determined with the Beckman pH meter model G at each temperature.

Assuming Lambert and Beer's law to hold, the molar percentage (f) of MHbCN in the sample can be represented by the following equation

$$f=\frac{\varepsilon_{MHb}-\varepsilon}{\varepsilon_{MHb}-\varepsilon_{MHbCN}}=\frac{a}{b} \qquad (1)$$

where ε_{MHb}, ε_{MHbCN} and ε stand for the optical densities at the selected wavelenth of MHb^+, MHbCN and their mixture, respectively, and a and b are the values of the optical density as shown in Fig. 1. All the measurements were made in the Soret region, where the absorption maxima of MHb^+ and MHbCN are 406 mμ and 420 mμ, respectively (*cf.* Fig. 1). The value f described above is also defined as follows:

$$f=\frac{[MHbCN]}{[MHb^+]+[MHbCN]} \qquad (2)$$

where $[MHb^+]$ and [MHbCN] are the concentration of free MHb^+ and of MHbCN, respectively. The over-all dissociation constant (K) of MHbCN is represented by

$$K=\frac{[MHb^+]\,[CN^-]}{[MHbCN]} \qquad (3)$$

where $[CN^-]$ is the concentration of free CN^- ions, which is dependent upon pH, the concentration of KCN, [KCN], and the dissociation constant (K_{HCN}) of HCN, namely:

$$[CN^-]=\frac{[KCN]}{\frac{[H^+]}{K_{HCN}}+1.} \qquad (4)$$

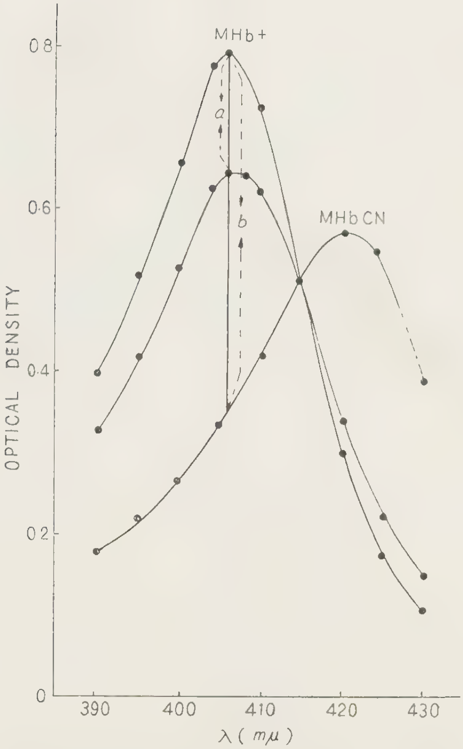

FIG. 1. Absorption spectra of MHb$^+$, MHbCN and their mixture at pH 6.80 and 16.5°

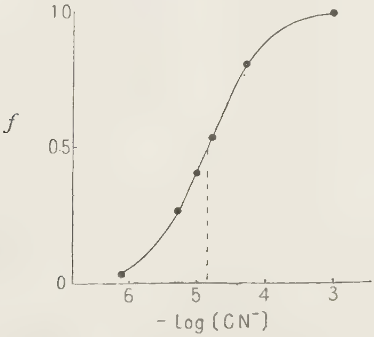

FIG. 2. The change of f, the molar percentage of MHbCN, as a function of log [CN$^-$]. In this case (pH 5.10, temp. 14°) the value of pK determined was 4.83.

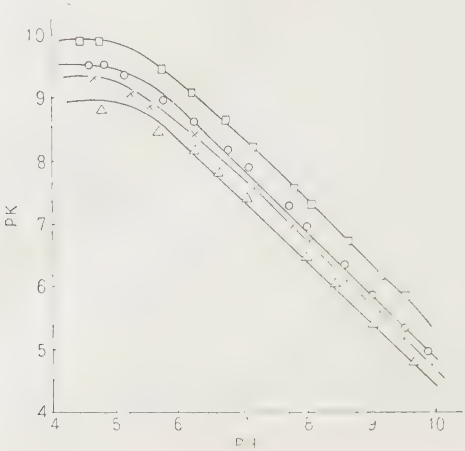

FIG. 3. The value of pK in function of pH at different temperatures.

□: 4°
○: 14°
×: 16.5°
△: 25°

Under the condition of our experiment, [MHbCN] is negligible compared with [CN^-], so that the following equation can be derived from Eqs. 2, 3 and 4:

$$K=\frac{1-f}{f}[CN^-]. \tag{5}$$

In accordance with this equation, the relation between f and log [CN^-] was found to be represented by a sigmoid curve of first order[*] as shown in Fig. 2, and the value of (CN^-) corresponding to $f={}^1/_2$ being equal to K. Making use of this fact pK was determined over the temperature range of 4–25° and the pH range of 4–10. The results are given in Fig. 3. The fact that pK values changes markedly with pH indicates that the equilibrium between MHb^+ and CN^- can not be represented by asingle formula such as

$$MHb^+ + CN^- \rightleftarrows MHbCN$$

Conceivably, MHb^+ has a hematin linked group which exerts an appreciable influence upon the equilibrium between MHb^+ and CN^-. We may consider that the following four reaction-steps are involved in the over-all reaction,

---

[*] This fact indicates that the hematin-hematin interaction in MHb^+ molecule can be neglected in this case.

$$MHb^+ + OH^- \overset{K_{OH}}{\rightleftarrows} MHbOH \quad (6)$$

$$MHb^+ + H^+ \overset{K_1'}{\rightleftarrows} H^+MHb^+ \quad (7)$$

$$H^+MHb^+ + CN^- \overset{K_{G1}}{\rightleftarrows} H^+MHbCN \quad (8)$$

$$MHb^+ + CN^- \overset{K_{G2}}{\rightleftarrows} MHbCN \quad (9)$$

where Eq. 6 represents the dissociation of alkaline methemoglobin, Eq. 7 that of acid MHb$^+$, namely the hematin linked group, Eq. 8 that of H$^+$MHbCN, and Eq. 9 that of MHbCN. The equilibrium constant of each of these reactions may be designated by K_{OH}, K_1', K_{G1} and K_{G2}, respectively. Assuming the occurrence of these four steps of dissociation, the over-all equilibrium constant is calculated to be as follows:

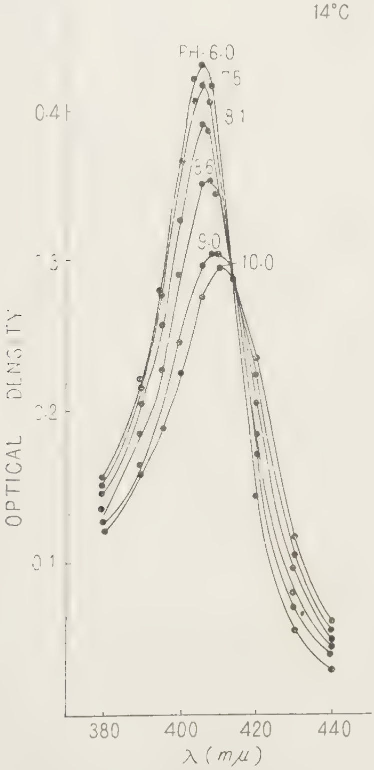

Fig. 4. Absorption spectrum of methemoglobin as it changed with pH

$$K=\left\{1+\frac{K_1'}{[H^+]}\left(1+\frac{K_W}{[H^+]}\cdot\frac{1}{K_{OH}}\right)\right\}\Big/\left(\frac{1}{K_{G1}}+\frac{K_1'}{[H^+]}\cdot\frac{1}{K_{G2}}\right) \quad (10)$$

where K_W is the ionic product of H_2O. $[H^+]$ can be determined directly by pH measurement and K_{OH} can be determined spectrophotometrically at each temperature. The results of measurement are given in Table I and Fig. 4. By applying the known values of K_W, K_{OH}, (H^+) and K (at 3 different pH values for each temperature) into equation 10, the values of K_1', K_{G1} and K_{G2} were calculated. On plotting the calculated values of pK_1', pK_{G1} and pK_{G2} against 1/T, it was found that all points fell on straight lines as shown in Fig. 5. From the tangent of these lines the enthalpies (ΔH) of the reactions can be estimated. The values obtained are presented in Table I.

TABLE I

Temp. C	4°	14°	16.5°	25°	ΔH (kcal)
pK_{OH}	8.98	8.64	8.56	8.30	+11.9
pK'_1	5.32	5.12	5.08	4.92	+7.2
pK_{G1}	9.90	9.50	9.40	9.10	+16.0
pK_{G2}	9.45	9.05	8.95	8.68	+16.0
pK_{G1}/pK_{G2}	0.45	0.45	0.45	0.42	

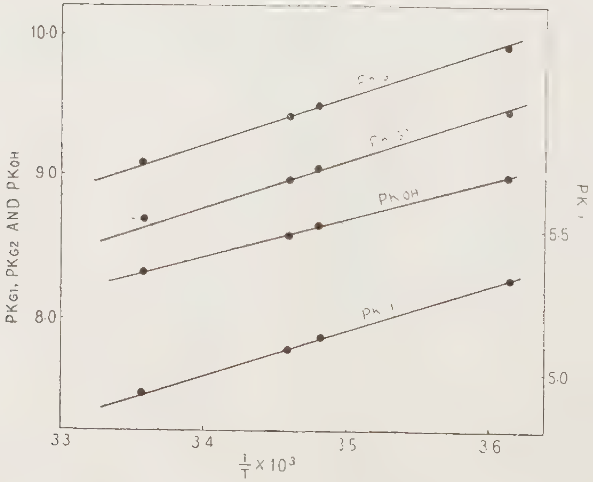

FIG. 5. Temperature dependency of pK'_1, pK_{G1}, pK_{G2} and pK_{OH}

In Fig. 3 the values of pK calculated by the above-mentioned values of K'_1, K_{G1} and K_{G2} in Eq. 10 are represented by curves and the corresponding experimental values are shown by points. As may be seen, the agreement between experimental and calculated values is quite satisfactory. From the temperature dependency of K'_1, the value of ΔH of the dissociation of H^+MHb^+ was estimated to be about 7.2 kcal. (*cf*. Table I).

Consideration should be given also to the dissociation of H^+MHbCN occurring in the manner:

$$H^+MHbCN \overset{K_2'}{\rightleftarrows} H^+ + MHbCN. \qquad (11)$$

where MHbCN represents in the cyan-methemoglobin molecule whose hematin linked group is dissociated. If the dissociation constant of this reaction is denoted by K'_2, we can derive from Eqs. 7, 8, 9 and 11 the following relation:

$$\frac{K_{G2}}{K_{G1}} = \frac{K_1'}{K_2'}. \qquad (12)$$

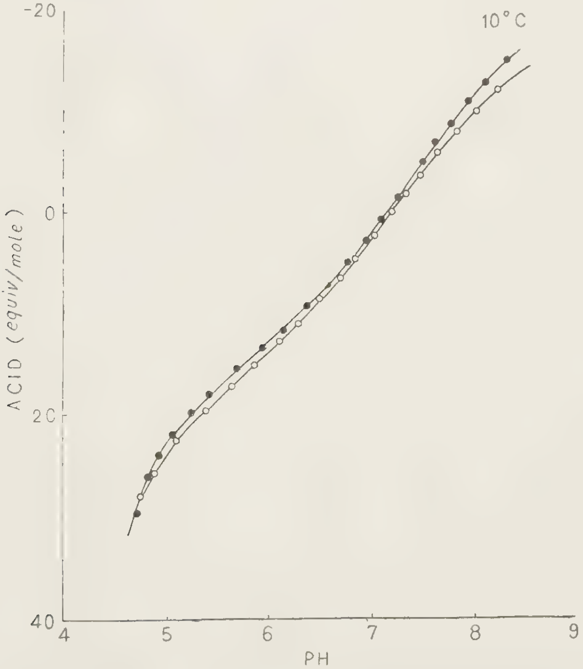

FIG. 6. Ttraition curves of MHb^+ and MHbCN at 10°

—●—●—●— MHb^+

—○—○—○— MHbCN

$[HCN]=10^{-2.1}$

Substituting in this equation, the values of K_{G1}, K_{G2} and K'_1 already known, pK'_2 at 25° was calculated to be 5.34. Since pK'_1 of the reaction $MHb^+ + H^+ \rightleftarrows H^+MHb^+$ was found to be 4.92, we may conclude that the coordination of CN^- to MHb^+ molecule had an influence of shifting the pK′ value of the hematin-linked group from 4.92 to 5.34.

Titrimetric Experiments

The stock solution of MHb^+ was diluted with distilled water until the ionic strength attained the value 0.5. To 240 ml. each of this solution was added 10 ml. of 0.5 *M* NaCN, on one hand, and 10 ml. of 0.5 *M* NaCl on the other. The con-

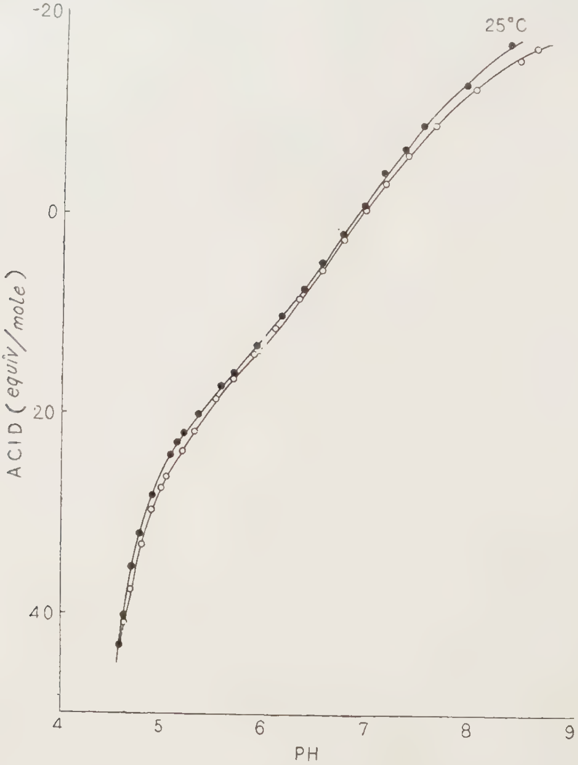

FIG. 7. Titration curves of MHb^+ and MHbCN at 25°

—●—●—●— MHb^+

—○—○—○— MHbCN

$[HCN] = 10^{-2.8}$

centrations of MHbCN or MHb^{+} in these solutions, which were in the range of 10^{-3}–10^{-4} mole per liter, were determined spectrophotometrically. The MHbCN solution was found to contain MHbCN and MHb^{+} in a ratio greater than 95 : 5 in the pH range studied (4.5–8.0), so that in the present calculation it was assumed that the concentration of MHbCN was equal to that of the total protein. Five ml. of the solution described above were titrated with 0.1 *N* HCl or NaOH using a microburette

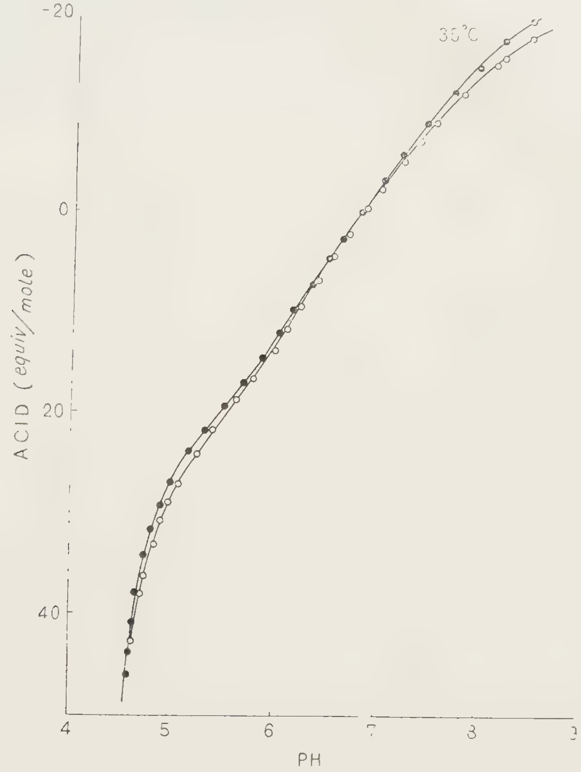

FIG. 8. Titration curves of MHb^{+} and MHbCN at 35°.
—●—●—●— MHb^{+}
—○—○—○— MHbCN
$[\text{HCN}]=10^{-2.1}$

and distilled water was added to make the total volume to 10 ml. The pH of the solution was measured in an oil bath thermostat by using Beckman glass electrode, which has been calibrated with Clark's buffers (*9*). Measurements were carried out at 10°, 25° and 35°.

The results are shown in Figs. 6–8, in which the equivalents of acid bound per mole protein present are plotted against pH. In the case of MHbCN, the equivalents of acid bound were calculated as the difference between the quantity of acid added and that of free HCN existed in the solution. Experiments were carried out several times at each temperature. At pH values below 5, the MHb^+ and MHbCN solutions showed some turbidity which, however, did not cause disturbances in the reproducibility of pH measurements. The following facts were brought out from these experiments. (i) At the pH value near 5, the difference (ΔA) between MHb^+ and MHbCN solutions in the quantity of acid bound per mole protein present was about 1 equivalent per mole protein (Fig. 9). (ii) The maximum value of ΔA was observed at pH 5.4 at 10°, at pH 5.1 at 25°, and at 4.9 at 35°. (iii) In all these cases the maximum values of ΔA was of the same magnitude, being about 1 equivalent per mole protein.

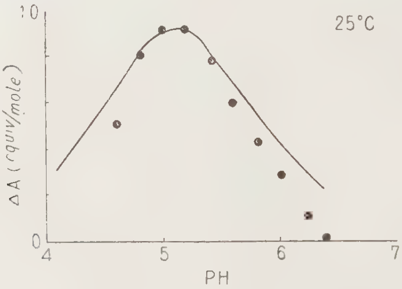

FIG. 9. The points show the observed values of ΔA, the difference between MHb^+ and MHbCN in the number of equivalents of acid bound per mole. The curve shows the values which were calculated on the basis of the explanation in text.

These findings may be explained by assuming the existence of one acid group for each hematin, whose dissociation constant shifts from pK'_1 to pK'_2 when the hematin in MHb^+ coordinates with CN^-. This assumption demands that the following equation should hold between ΔA and $[H^+]$:

$$\Delta A=4\left\{\frac{[H^+]}{K'_2+[H^+]}-\frac{[H^+]}{K'_1+[H^+]}\right\}.$$

By using the experimental values of ΔA and (H^+), the values of K'_1 and K'_2 which satisfy the above equation were estimated. The results obtained are shown in Table II. These results show that the pK′ value of the hematin linked group increases by 0.4 when hematin in MHb^+ combines with CN^-. The temperature dependency of pK'_1 and pK'_2 was found to be the same, both corresponding to the ΔH value of about 8 kcal.

TABLE II

Temp. C	10°	25°	35°
pK'_1	5.2	4.9	4.7
pK'_2	5.6	5.3	5.1

Titrimetric experiments performed at pH values above 7 showed that in these alkaline regions there occurred the formation of alkaline methemoglobin (MHbOH) accompanied by the dissociation of free HCN. In these alkaline regions, a great deal of NaCN was required to keep the ratio of MHbCN to MHbOH above 95 : 5, so that it was impossible to extend the titration curves to more alkaline regions.

DISCUSSION AND SUMMARY

As may be seen, the spectrophotometric and titrimetric investigations described above have yielded results which harmonize satisfactorily with each other. Both were accounted for by the assumption that (i) in MHb^+ molecule there is one dissociation group linked to each hematin, showing a pK′ value of about 4.9 at 25°, that (ii) the pK′ of this group shifts from 4.9 to 5.3 when MHb^+ combines with CN^-, and that (iii) the calculated value of the heat of dissociation of this group is 7–8 kcal. These results seem to suggest that the hematin linked group in question is most probably the imsdazole group of histidine, whose pK′ value in free histidine molecule is known to be about 6.0. The somewhat smaller values we have observed may be interpreted as being due to the interaction between the hematin and the linked group. If this assumption be correct, our experiment has revealed that when hematin combines with CN^-, the interaction in question is diminished and the pK′ value approaches that of the free imidazole.

It should be remarked that the pK′ value of hematin-linked group we have determined by an analysis of equilibrium between MHb^+ and CN^- coincides well with corresponding values found by other anothers using different methods: namely $pK'=5.45$ at 20° and 5.75 at 25° determined titrimetrically by Theorell (*4*) and Wyman (*5*), respectively, 5.3 ± 0.2 determined magnetometrically by Coryell and Pauling (*6*), and 5.1 at 20° estimated spectrophotometrically by George and Hanania (*7*).

The authors wish to express their sincere gratitude to Prof. H. Tamiya for his advices in this work. The pH measuring circuit was designed under the direction of Dr. S. Koga to whom we wish to acknowledge our indebtedness.

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LETTERS TO THE EDITORS

REMARQUES SUR LES PTÉRINES NATURELLES

Messieurs :

T. Hama et ses collaborateurs ont publié récemment une série de communications sur les ptérines naturelles (*1*, *2*). Cependant quelques uns de leurs résultats sont contradictoires avec les nôtres et leurs interprétations ne nous semblent pas toujours justes. C'est pourquoi nous tenons à présenter ici les recents résultats de nos recherches sur les ptérines naturelles en les comparant avec ceux décrits par les autres chercheurs.

Nous avons extrait une substance avec la fluorescence violette à partir des écailles de carpe, *Cyprinus carpio* et *Cyprinus auratus* (*3*), cependant nous avons trouvé qu'elle était un mélange de trois ptérines différentes. Plus tard nous avons séparé les trois ptérines et en comparant les valeurs du Rf en divers solvants, les attitudes à la réduction par Al-Hg (*4*), à l'oxydation par MnO_2 (*4*) et à l'estérification par étanol-HCl des trois ptérines avec celles des ptérines synthétisées, nous avons mis en évidence que deux de trois sont l'*iso*xanthoptérine et l'acide *iso*xanthoptérine-carboxylique respectivement. L'une des trois, qui reste, est réduite en l'*iso*xanthoptérine par Al-Hg et oxydée en l'acide *iso*xanthoptérine-carboxylique par MnO_2.

Dr. R. Hüttel a eu la bonté de nous procurer des écailles de *Leuciscus rutilus*, qui'l avait employées pour l'extraction d'ichtyoptérine (*5*). Nous en avons isolé trois ptérines (fluorescyanine A_2, d'après Busnel *et al.*[*]) tout à fait identiques à celles isolées de la carpe japonaise, *Cyprinus carpio* et *Cyprinus auratus* (fluorescyanine A_1, d'après Busnel *et al.*[*]). La ptérine, que Hüttel a nommée l'ichtyoptérine, est montrée identique à notre troisième ptérine, qui peut être réduite en l'*iso*xanthoptérine par Al-Hg.

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[*] Busnel, R. G. et Jérôme, H., (*J. Biochem.*, **42**, 363 (1955)), ont proposé le nom génerique "fluorescyanine" pour les substances avec la fluorescence bleue mais nous ne sommes pas tout à fait d'accord de unifier des ptrénes sous le nom "fluorescyanine", parce qu'il est incommode; souvent on ne peut pas distinguer. les ptérines par l'observation de leur "couleurs" de la fluorescence. Par notre recherche presenté ci-dessus, il est evident que la fluorescyanine A_1 est identique à la fluorescyanine A_2.

R. Tschesche et F. Korte ont donné la constitution l'acide 2-amino-4, 7-dioxyptéridyl-6-acétique (**I**) à l'ichtyoptérine (*6*). Cependant nous avons montré que (**I**) à la valeur du Rf différente de l'ichtyoptérine en solvant, sodium citrique à 4 pour cent. De plus (**I**) est très facilement décarboxylé en 6-methylisoxanthoptérine, n'est pas réduit par Al-Hg, et peut être estérifié par étanol-HCl. Tous les caractères cidessus ne coincident pas avec ceux de l'ichtyoptérine. Donc nous croyons que l'ichtyoptérine n'est pas identique à (**I**).

K. Kushibiki, T. Hama et T. Gotô ont nommé les ptérines dans les écailles de carpe cyprino-pourpre A_1, cyprino-pourpre A_2 et cyprino-pourpre B (*2*). En réalité ils sont identique à *iso*xanthoptérine, ichtyoptérine et acide isoxanthoptérine-carboxylique respectivement. D'après eux, le cyprino-pourpre A_2 est transformé en cyprino-pourpre B par les rayons solaires (*2*), mais nous ne pouvans pas le confirmer. Seulement l'ichtyoptérine perd sa fluorescence par irradiation prolongée.

Autrefois nous avons extrait une ptérine à partir des oeufs et des larves fraîchement écloses de *Bombyx mori* et l'avons nommée leucoptérine-B (7), cependant plus tard nous avons confirmé que ce n'était que l'*iso*xanthoptérine (*8*).

D'autre part, nous avons isolé trois ptérines à partir de la peau de *Rana nigromaculata*; soit, l'*iso*xanthoptérine, l'acide 2-amino-4-oxy-ptéridine-6-carboxylique (**II**) et une ptérine inconnue, qui est transformée en (**II**) par les rayons solaires ou par MnO_4K, mais pas par Al-Hg.

T. Hama a nommé les substances fluorescentes obtenues de la peau et des yeux de *Rana nigromaculata* rana-chromes 1, 2, 3, 4, 4B, 5 et 6. D'après lui, le rana-chrome 5 a la constitution (**II**) et il est obtenu par l'action du rayonnement lumineux sur le rana-chrome 1, et le ranachrome 4 serait l'ichtyoptérine ou une ptérine très proche de l'ichtyoptérine ou de la fluorescyanine, qui a le spectre d'absorption différente de celui de l'*iso*xanthoptérine (*1*, *2*, *10*). Les trois ptérines, que nous avons isolées, correspondent aux rana-chromes 1, 4 et 5. Nous considérons que le rana-chrome 4 de Hama, étant impure, a le spectre d'absorption un peu différent de celui d'isoxanthoptérine.

Nous tenons à remercier M. le Prof. F. Egami des conseils précieux et M. le Dr. R. Hüttel de la bonté de nous avoir procuré des échantillons.

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(Reçu, le 29 Mars, 1955)

REMARQUES SUR LA NOMENCLATURE DES PTÉRINES NATURELLES

Messieurs :

Les notes récemment présentées à la Socitété Franco-Japonaise de Biologie par diverses équipes de chercheurs japonais sur les ptérines naturelles (1951—1954) (*1*) nous amènent à faire quelques remarques concernant la nomenclature utilisée.

La complexité et la répartition zoologique étendue des ptérines naturelles hydrosolubles que nous sommes parmi les premiers à avoir singnalées (1938—1948) (*2*) à la fois chez les Vertébrés inférieurs, les Crustacés et les Insectes, nous ont amené à les grouper sous le vocable d'ensemble de " substances à fluorescence bleue ". En 1942 (*2 bis*), à l'occasion de nos recherches sur les tubes de Malpighi des Insectes, nous en décrivions déjà deux types, *α* et *β*.

Certes, ces termes étaient fort imprécis. Ils avaient cependant une origine fort ancienne ; Bence Jones (*3*) les a, probablement le premier, décrites dans ce qu'il nommait " quinoïdine animale ", par analogie de la fluorescene avec celle de la quinine ; il s'agissait de tout un groupe d'extraits de tissus animaux comportant probablement, entre autre, des extraits ptériniques avant la lettre. Ce terme fut repris en 1925 par Kinnersley, Peters et Squires (*4*); ils proposèrent de désigner comme " quinochromes " toutes les substances incolores et non déterminées, fluorescentes en bleu et mises ainsi en évidence. C'est à von Euler et Adler (1934) (*5*) que l'on doit pour la première fois le terme de " substances à fluorescence bleue ".

Ce n'est que dès que l'une de ces substances à fluorescence bleue, décrite dans nos tous premiers travaux, fut isolée, en 1943, qu'avec Polonovski et Pesson (*6*) nous couvrions l'ensemble de ces composés sous le nom générique de " fluorescyanine ", terme définissant bien l'une de leurs propriétés essentielles, " la fluorescence bleue ", celle décrite étant isolée d'une seule espèce animale, *Cyprinus carpio* L.

A la même époque, Hüttel et Sprengling (1943) (*7*), en s'appuyant d'ailleurs sur nos travaux antérieurs, arrivent, en partant d'un mélange d'écailles de plusieurs espèces de Poissons, à l'isolement de l'une de cse substances et qui actuellement ne paraît pas identique à celle que nous avons décrite chez la Carpe—(voir Korte (1954)) (*8*): ils proposèrent de nommer ce composé " ichtyoptérine ".

Si ce terme avait le mérite d'introduire la notion de " ptérine ", il nous paraîssait trop étroitement rattaché à un groupe zoologique.

En effet, quelques temps après, nous caractérisions une autre fluorescyanine dans les oeufs et les ailes de *Bombyx mori* L. (1948) (*9*) et ceci nous amenait à confirmer les raisons de notre nomenclature : celle-ci fut proposée et adoptée au cours d'un Symposium International tenu à Paris en 1952, où se trouvait un représentant Japonais, le Professeur Egami.

Il est bien évident que ce terme générique de fluorescyanine ne peut couvrir ces substances que d'une manière provisoire, jusqu'à ce que la nature chimique des produits soit définitivement établie.

Dans l'état actuel de nos connaissances, il nous paraîssait possible de regrouper sous ce vocable de fluorescyanine, toutes les substances ptériniques hydrosolubles à fluorescene bleue, la lettre A étant valable pour tous les Vertébrés inférieurs et la lettre B pour les Invertébrés. Les indications complémentaires A_1, A_2, A_3 déterminant soit une origine particulière, soit un rF, ainsi qu'on a l'habitude de le faire en biochimie pour des corps non encore complètement définis (Vitamine A_1, A_2, B_1, B_2 : Cytochromes A_1, A_2, *etc*....). Nous rappelons d'ailleurs l'abandon relativement rapide de termes ayant un radical rappelant l'origine des corps, au fur et à mesure qu'on les découvrait ailleurs, par exemple la " Lactoflavine ", et l'on avait proposé au Symposium de nommer :
—Fluorescyanine A_1, la fluorescyanine du mélanocyte de Carpe, décrite par Polonovski, Busnel et Pesson;
—Fluorescyanine A_2, l'Ichtyoptérine d'écailles de poissons décrite par Hüttel, la différence reposant sur les rF et l'origine des produits;
—Fluorescyanine B_1, la fluorescyanine de l'oeuf de *B. mori*, décrite par Polonovski, Busnel et Pesson; *etc.*, *etc*....

Dans le domaine étroit de nos pigments ptéridiniques, nous discuterons les propositions japonaises telles qu'elles découlent des travaux de ces dernières années.

Les termes de cyprino-pourpre et de rana-chrome (Kushibiki, Hama et Goto—1954) (*1*) nous paraîssent particulièrement impropres. Pour le premier, le pourpre est une couleur et le pigment est incolore ; pour le second, le terme chrome signifie encore couleur et le pigment est incolore à la lumière blanche. En outre, l'emploi du terme rana-chrome pour la ptérine du Crustacé (*Cambarus Clarkii*) est un bon exemple de l'inadaptation du terme zoologique.

En ce qui concerne la Grenouille (*Rana*), il est nécessaire de rappeler que les premières observations connues sur les substances fluorescentes de sa peau et de ses divers tissus reviennent à un histologiste, élève du Prof. Policard, Hadjioloff (1929) (*10*), qui retrouva également une forte fluorescence dans les cendres de ces mêmes organes. Pour notre part, nous avons confirmé à plusieurs reprises qu'il y avait des substances à fluorescence bleue chez les Batraciens (1942) (*2*) et de la fluorescyanine (1943) (*2*), (1945) (*11*).

Certes, un caractère de fluorescence ne peut définir les propriétéé chimiques d'un corps, mais, tant que les biochimistres n'auront pas réussi à caractériser la nature réelle des différents composés en présence, nous sommes persuadés qu'il y a intérêt à ce que le vocable tende vers une unification, et ce, d'une manière logique; l'adoption d'un terme considéré comme générique, tel que "fluorescyanine" historiquement dérivé des substances à fluorescene bleue, devrait pouvoir provisoirement rallier tous les auteurs, biologistes ou biochimistes, oeuvrant parallèlement dans ce domaine, ce qui faciliterait grandement, même pour les spécialistes, la précision nécessaire à la bonne marche de telles recherches.

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